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Substitute for PTO/SB/05 (08/00) Utility Patent Application Transmittal  
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# UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. SD-99511A

First Inventor or Application Identifier A. GILLESPIE ET AL.

Title DNA ENCODING HUMAN  $\alpha$  AND  $\beta$  SUBUNITS...RECEPTOR

Express Mail Label No. EL523906834US

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09/703951

11/01/00

APPLICATION ELEMENTS <i>See MPEP chapter 600 concerning utility patent application contents.</i>		ADDRESS TO: Assistant Commissioner for Patents Box Patent Application Washington, DC 20231	
1. <input checked="" type="checkbox"/> Fee Transmittal Form (Submit an original, and a duplicate for fee processing)		6. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)	
2. <input checked="" type="checkbox"/> Specification [ Total Pages <u>54</u> ]		a. <input type="checkbox"/> Computer Readable Form	
3. <input checked="" type="checkbox"/> Drawing(s) (35 USC 113) [ Total Sheets <u>14</u> ] [ Total Pages <u>68</u> ]		b. <input checked="" type="checkbox"/> Paper Copy (identical to computer copy)	
		c. <input type="checkbox"/> Statements verifying identity of above copies	
4. Oath or Declaration <b>UNEXECUTED</b> a. <input checked="" type="checkbox"/> Newly executed (original or copy) b. <input type="checkbox"/> Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional with Box 14 completed)  i. <input type="checkbox"/> <b>DELETION OF INVENTOR(S)</b> Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33 (b).		<b>ACCOMPANYING APPLICATION PARTS</b> 7. <input type="checkbox"/> Assignment papers (cover sheet & document(s)) 8. <input type="checkbox"/> 37 CFR 3.73(b) Statement (when there is an assignee) <input type="checkbox"/> Power of Attorney 9. <input type="checkbox"/> Information Disclosure Statement (IDS)/PTO-1449 <input type="checkbox"/> Copies of IDS Citations 10. <input type="checkbox"/> Preliminary Amendment 11. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) (Should be specifically itemized) 12. <input type="checkbox"/> Certified Copy of Priority Document(s) (if foreign priority is claimed) 13. <input type="checkbox"/> Other: _____	
5. <input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76			
14. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76: <input type="checkbox"/> Continuation <input type="checkbox"/> Divisional <input checked="" type="checkbox"/> Continuation-in-part (CIP) of prior application No. <u>08 / 487,596</u> Prior application information: Examiner <u>WESLEY E. LAZAR</u> Group/Art Unit: <u>1646</u> For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference.			
15. CORRESPONDENCE ADDRESS			
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EXPRESS MAIL CERTIFICATE	
DATE OF DEPOSIT	<u>November 1, 2000</u>
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## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents  
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Deposit Acct. 13-2755  
MERCK & CO., INC.  
Our Case Docket No. SD99511A

Sir:

Transmitted herewith for filing under 37 C.F.R. §1.53(b) is the patent application of Inventor(s):  
ALISON GILLESPIE, BRIAN O. CLAEPS, LAURA ELENA CHAVEZ-NORIEGA, ROBERT SIEGEL AND KATHRYN J. ELLIOTT

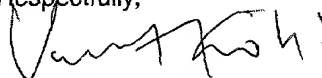
For: DNA ENCODING HUMAN  $\alpha$  AND  $\beta$  SUBUNITS OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR, CELLS TRANSFORMED THEREWITH, AND RECOMBINANT CELL LINE EXPRESSING A HUMAN  $\alpha$  AND  $\beta$  SUBUNIT OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR

For	Number Filed	Number Extra	Rate	Basic Fee \$710
Total Claims	13 - 20 =	0 X	\$18	= \$0
Independent Claims	3 - 3 =	0 X	\$80	= \$0
Multiple Dependent Claims*		X	\$270	=
* Add this fee if application contains any multiple dependent claims, regardless of number.		TOTAL FILING FEE →		\$710

Please charge my Deposit Account No. 13-2755 in the amount of \$ 710 . The Assistant Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Account No. 13-2755. Duplicate copy of this sheet is enclosed.

☒ Under the provisions of 37 C.F.R. §1.53, this application is being filed without the declaration of each inventor.

Respectfully,



By: VINEET KOHLI

Attorney For Applicant(s)

Reg. No. 37,003

MERCK & CO., INC.

Patent Dept., RY60-30

P.O. Box 2000

Rahway, N.J. 07065-0907

(732) 594-3889

Date: November 1, 2000

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MAILED BY	<u>Jode Hobbs</u> DATE <u>11/1/2000</u>

IN DUPLICATE

DNA encoding human  $\alpha$ - and  $\beta$ -subunits of neuronal nicotinic acetylcholine receptor, cells transformed therewith, and a recombinant cell line expressing a functional human  $\alpha_7$  subunit of neuronal nicotinic acetylcholine receptor

This application is a continuation-in-part of U.S. application Serial No. 08/487,596 filed 6/7/95, now pending, which is a continuation-in-part of U.S. application Serial No. 08/149,503, filed November 8, 1993, now pending; and a continuation-in-part of U.S. application Serial No. 08/028,031, filed March 8, 1993, now abandoned; and a continuation-in-part of U.S. application Serial No. 07/938,154, filed November 30, 1992, which is a continuation-in-part of U.S. application Serial No. 07/504,455, filed April 3, 1990, now issued as U.S. Letters Patent No. 5,369,028, each of which is hereby incorporated by reference herein in their entirety.

This invention relates to DNA encoding human neuronal nicotinic acetylcholine receptor protein subunits, as well as the proteins themselves. In particular, human neuronal nicotinic acetylcholine receptor  $\alpha$ -subunit-encoding DNA,  $\alpha$ -subunit proteins,  $\beta$ -subunit-encoding DNA,  $\beta$ -subunit proteins, and combinations thereof are provided. A non-human cell line expressing a human  $\alpha$ -subunit protein is also disclosed.

## BACKGROUND OF THE INVENTION

Ligand-gated ion channels provide a means for communication between cells of the central nervous system. These channels convert a signal (e.g., a chemical referred to as a neurotransmitter) that is released by one cell into an electrical signal that propagates along a target cell membrane. A variety of neurotransmitters and neurotransmitter receptors exist in the central and peripheral nervous systems. Five families of ligand-gated receptors, including the nicotinic acetylcholine receptors (NACHRs) of neuromuscular and neuronal origins, have been identified (Stroud et al. (1990) Biochemistry 29:11009-11023). There is, however, little understanding of the manner in which the variety of receptors generates different responses to neurotransmitters or to other modulating ligands in different regions of the nervous system.

The nicotinic acetylcholine receptors (NACHRs) are multisubunit proteins of neuromuscular and neuronal origins. These receptors form ligand-gated ion channels that mediate synaptic transmission between nerve and muscle and between neurons upon interaction with the neurotransmitter acetylcholine (ACh). Since various nicotinic acetylcholine receptor (NACHR) subunits exist, a variety of NACHR compositions (i.e., combinations of subunits) exist. The different NACHR compositions exhibit different specificities for various ligands and are thereby pharmacologically distinguishable. Thus, the nicotinic acetylcholine receptors expressed at the vertebrate neuromuscular junction in vertebrate sympathetic ganglia and in the vertebrate central nervous system have been distinguished on the basis of the effects of various ligands that bind to different NACHR compositions. For example, the elapid  $\alpha$ -neurotoxins that block activation of nicotinic acetylcholine receptors at the neuromuscular junction do not block activation of some neuronal nicotinic acetylcholine receptors that are expressed on several different neuron-derived cell lines.

Muscle NACHR is a glycoprotein composed of five subunits with the stoichiometry  $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$ . Each of the subunits has a mass of about 50-60 kilodaltons (kd) and is encoded by a different gene. The  $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$  complex forms functional receptors containing two ligand binding sites and a ligand-gated transmembrane channel. Upon interaction with a cholinergic agonist, muscle nicotinic AChRs conduct sodium ions. The influx of sodium ions rapidly short-circuits the normal ionic gradient maintained across the plasma membrane, thereby depolarizing the membrane. By reducing the potential difference across the membrane, a chemical signal is transduced into an electrical signal that signals muscle contraction at the neuromuscular junction.

Functional muscle nicotinic acetylcholine receptors have been formed with  $\alpha\beta\delta\gamma$  subunits,  $\alpha\beta\gamma$  subunits,  $\alpha\beta\delta$  subunits,  $\beta\delta\gamma$  subunits or  $\alpha\delta$  subunits, but not with only one subunit (see e.g., Kurosaki et al. (1987) FEBS Lett. 214:253-258; Camacho et al. (1993) J. Neuroscience 13:605-613). In contrast, functional neuronal AChRs (nAChRs) can be formed from  $\alpha$  subunits alone or combinations of  $\alpha$  and  $\beta$  subunits. The larger  $\alpha$  subunit is generally believed to be the ACh-binding subunit and the lower molecular weight  $\beta$  subunit is generally believed to be the structural subunit, although it has not been definitively demonstrated that the  $\beta$  subunit does not have the ability to bind ACh. Each of the subunits which participate in the formation of a functional ion channel are, to the extent they contribute to the structure of the resulting channel, "structural" subunits, regardless of their ability (or inability) to bind ACh. Neuronal AChRs (nAChRs), which are also ligand-gated ion channels, are expressed in ganglia of the autonomic nervous system and in the central nervous system (where they mediate signal transmission), in post-synaptic locations (where they modulate transmission), and in pre- and extra-synaptic locations (where they may have additional functions).

DNA encoding NACHRs has been isolated from several sources. Based on the information available from such work, it has been evident for some time that NACHRs expressed in muscle, in autonomic ganglia, and in the central nervous system are functionally diverse. This functional diversity could be due, at least in part, to the large number of different NACHR subunits which exist. There is an incomplete understanding, however, of how (and which) NACHR subunits combine to generate unique NACHR subtypes, particularly in neuronal cells. Indeed, there is evidence that only certain NACHR subtypes may be involved in diseases such as Alzheimer's disease. Moreover, it is not clear whether NACHRs from analogous tissues or cell types are similar across species.

Accordingly, there is a need for the isolation and characterization of DNAs encoding each human neuronal NACHR subunit, recombinant cells containing such subunits and receptors prepared therefrom. In order to study the function of human neuronal AChRs and to obtain disease-specific pharmacologically active agents, there is also a need to obtain isolated (preferably purified) human neuronal nicotinic AChRs, and isolated (preferably purified) human neuronal nicotinic AChR subunits. In addition, there is also a need to develop assays to identify such pharmacologically active agents.

The availability of such DNAs, cells, receptor subunits and receptor compositions will eliminate the uncertainty of speculating as to human nNACHR structure and function based on predictions drawn from non-human nNACHR data, or human or non-human muscle or ganglia NACHR data.



Therefore, it is an object herein to isolate and characterize DNA encoding subunits of human neuronal nicotinic acetylcholine receptors. It is also an object herein to provide methods for recombinant production of human neuronal nicotinic acetylcholine receptor subunits. It is also an object herein to provide purified receptor subunits and to provide methods for screening compounds to identify compounds that modulate the activity of human neuronal AChRs.

Likewise, it is an object of the present invention to provide a recombinant non-human cell line transformed with a heterologous nucleic acid molecule that encodes a human  $\alpha$  subunit of neuronal nAChR.

These and other objects will become apparent to those of skill in the art upon further study of the specification and claims.

### BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided isolated DNAs encoding novel human alpha and beta subunits of neuronal NACHRs. Also provided is a non-human cell line that expresses a human  $\alpha_7$  subunit of neuronal nAChR. In particular, isolated DNA encoding human  $\alpha_4$ ,  $\alpha_7$ , and  $\beta_4$  subunits of neuronal NACHRs are provided. Messenger RNA and polypeptides encoded by the above-described DNA are also provided.

Further in accordance with the present invention, there are provided recombinant human neuronal nicotinic AChR subunits, including  $\alpha_4$ ,  $\alpha_7$ , and  $\beta_4$  subunits, as well as methods for the production thereof. In addition, recombinant human neuronal nicotinic acetylcholine receptors containing at least one human neuronal nicotinic AChR subunit are also provided, as well as methods for the production thereof. Further provided are recombinant neuronal nicotinic AChRs that contain a mixture of one or more NACHR subunits encoded by a host cell, and one or more nNACHR subunits encoded by heterologous DNA or RNA (i.e., DNA or RNA as described herein that has been introduced into the host cell), as well as methods for the production thereof.

Plasmids containing DNA encoding the above-described subunits are also provided. Recombinant cells containing the above-described DNA, mRNA or plasmids are also provided herein. Such cells are useful, for example, for replicating DNA, for producing human NACHR subunits and recombinant receptors, and for producing cells that express receptors containing one or more human subunits.

Also provided in accordance with the present invention are methods for identifying cells that express functional nicotinic acetylcholine receptors. Methods for identifying compounds which modulate the activity of NACHRs are also provided. Invention methods employ that isolated DNAs, encoding human  $\alpha$  and  $\beta$  subunits of neuronal AChRs and polypeptides encoded thereby.

The DNA, mRNA, vectors, receptor subunits, receptor subunit combinations and cells provided herein permit production of selected neuronal nicotinic AChR receptor subtypes and specific combinations thereof, as well as antibodies to said receptor subunits. This provides a means to prepare synthetic or recombinant receptors and receptor subunits that are substantially free of

contamination from many other receptor proteins whose presence can interfere with analysis of a single NACHR subtype. The availability of desired receptor subtypes makes it possible to observe the effect of a drug substance on a particular receptor subtype and to thereby perform initial *in vitro* screening of the drug substance in a test system that is specific for humans and specific for a human neuronal nicotinic AChR subtype.

The availability of subunit-specific antibodies makes possible the application of the technique of immunohistochemistry to monitor the distribution and expression density of various subunits (e.g., in normal vs diseased brain tissue). Such antibodies could also be employed for diagnostic and therapeutic applications.

The ability to screen drug substances *in vitro* to determine the effect of the drug on specific receptor compositions should permit the development and screening of receptor subtype-specific or disease-specific drugs. Also, testing of single receptor subunits or specific receptor subtype combinations with a variety of potential agonists or antagonists provides additional information with respect to the function and activity of the individual subunits and should lead to the identification and design of compounds that are capable of very specific interaction with one or more of the receptor subunits or receptor subtypes. The resulting drugs should exhibit fewer unwanted side effects than drugs identified by screening with cells that express a variety of subtypes.

Further in relation to drug development and therapeutic treatment of various disease states, the availability of DNAs encoding human nNACHR subunits enables identification of any alterations in such genes (e.g., mutations) which may correlate with the occurrence of certain disease states. In addition, the creation of animal models of such disease states becomes possible, by specifically introducing such mutations into synthetic DNA sequences which can then be introduced into laboratory animals or *in vitro* assay systems to determine the effects thereof.

#### DETAILED DESCRIPTION OF THE FIGURES

FIG. 1 presents a restriction map of two pCMV promoter-based vectors, pCMV-T7-2 and pCMV-T7-3.

FIG. 2 presents a restriction map of a pCMV promoter based vector, pcDNA3-KE $\alpha_7$ RBS.

FIG. 3 depicts the nicotine and acetylcholine –induced dose-response curves for the A7 cell line obtained from functional bulk calcium assays.

FIG. 4 depicts the kinetics of the A7 stable cell line obtained by electrophysiological analysis.

FIG. 5 depicts the MLA and  $\alpha$ -bungarotoxin (ligands of A7) binding assay of A7.

FIG. 6 depicts the results of a single cell calcium imaging of the A7 cell line, showing the homogeneity of the response of the A7 cell line to acetylcholine.

FIG. 7 depicts the results of a Western blot analysis using an A-7 specific antibody as a probe. The data specifically confirm expression of the  $\alpha_7$  protein by the A7 cells.

FIG. 8 shows the results of a Northern Blot analysis of total RNA prepared from A7 cells.

FIG. 9 a - b compares agonist-induced dose-response curves of the alpha3beta2alpha5 expressing cells and alpha3beta2 expressing cells and specifically shows that their profile differs from that of an alpha3beta2 subunit combination.

FIG. 10 depicts a comparison in the kinetics of decay of currents induced by acetylcholine between A3B2A5 cells and A3B2 cells.

FIG. 11 confirms the association of the alpha3 and beta 2 with alpha 5 subunits in cell line A3B2A5.

FIG. 12 depicts the expression construct for alpha 3 - -pc DNA3-KEalpha3

FIG. 13 depicts the expression construct for alpha 5 - -pHook3-KEalpha5RBS

FIG. 14 depicts the expression construct for beta 2-- pc DNA3-KEbeta2RBS

## DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have isolated and characterized DNAs encoding novel human alpha and beta subunits of neuronal nAChR. Specifically, isolated DNAs encoding human  $\alpha_4$ ,  $\alpha_7$ , and  $\beta_4$  subunits of neuronal Anchors are described herein. Recombinant messenger RNA (mRNA) and recombinant polypeptides encoded by the above-described DNA are also provided.

In accordance with the present invention, we have developed methods for identifying compounds that modulate the activity of nAChRs, which employ DNAs encoding human  $\alpha$  and  $\beta$  subunits of neuronal nAChRs and polypeptides encoded thereby. Specifically, screening methods employing DNAs encoding human  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_6$ ,  $\alpha_7$ ,  $\beta_2$ ,  $\beta_3$ ,  $\beta_4$ , subunits of neuronal NACHRs is described herein.

Also described are isolated cells experiencing various multimeric combinations of human  $\alpha$  and  $\beta$  subunits of neuronal nAChRs, i.e., 3-, 4- and 5-way combinations. A non-human cell line expressing human  $\alpha_7$  subunit is also described herein.

As used herein, isolated (or substantially pure) as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their in vivo cellular environments through the efforts of human beings. Thus as used herein, isolated (or substantially pure) DNA refers to DNAs purified according to standard techniques employed by those skilled in the art (see, e.g., Maniatis et al. (1982) *Molecular*

*Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Similarly, as used herein, "recombinant" as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been prepared by the efforts of human beings, e.g., by cloning, recombinant expression, and the like. Thus as used herein, recombinant proteins, for example, refers to proteins produced by a recombinant host, expressing DNAs which have been added to that host through the efforts of human beings.

As used herein, a human alpha subunit gene is a gene that encodes an alpha subunit of a human neuronal nicotinic acetylcholine receptor. The alpha subunit is a subunit of the nAChR to which ACh binds. Assignment of the name "alpha" to a putative nNACHR subunit, according to Deneris et al. [Tips (1991) 12:34-40] is based on the conservation of adjacent cysteine residues in the presumed extracellular domain of the subunit that are the homologues of cysteines 192 and 193 of the Torpedo alpha subunit (see Noda et al. (1982) *Nature* 299:793-797). As used herein, an alpha subunit subtype refers to a human nNACHR subunit that is encoded by DNA that hybridizes under high stringency conditions to at least one of the nNACHR alpha subunit-encoding DNAs (or deposited clones) disclosed herein. An alpha subunit also binds to ACh under physiological conditions and at physiological concentrations and, in the optional presence of a beta subunit (i.e., some alpha subunits are functional alone, while others require the presence of a beta subunit), generally forms a functional AChR as assessed by methods described herein or known to those of skill in this art.

Also contemplated are alpha subunits encoded by DNAs that encode alpha subunits as defined above, but that by virtue of degeneracy of the genetic code do not necessarily hybridize to the disclosed DNA or deposited clones under specified hybridization conditions. Such subunits also form a functional receptor, as assessed by the methods described herein or known to those of skill in the art, generally with one or more beta subunit subtypes. Typically, unless an alpha subunit is encoded by RNA that arises from alternative splicing (i.e., a splice variant), alpha-encoding DNA and the alpha subunit encoded thereby share substantial sequence homology with at least one of the alpha subunit DNAs (and proteins encoded thereby) described or deposited herein. It is understood that DNA or RNA encoding a splice variant may overall share less than 90% homology with the DNA or RNA provided herein, but include regions of nearly 100% homology to a DNA fragment or deposited clone described herein, and encode an open reading frame that includes start and stop codons and encodes a functional alpha subunit.

As used herein, a splice variant refers to variant NACHR subunit-encoding nucleic acid(s) produced by differential processing of primary transcript(s) of genomic DNA, resulting in the production of more than one type of mRNA. cDNA derived from differentially processed genomic DNA will encode NACHR subunits that have regions of complete amino acid identity and regions having different amino acid sequences. Thus, the same genomic sequence can lead to the production of multiple, related mRNAs and proteins. Both the resulting mRNAs and proteins are referred to herein as "splice variants".

Stringency of hybridization is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature ( $T_m$ ) of the hybrids.  $T_m$  can be approximated by the formula:

$$81.5^{\circ}\text{C.} - 16.6(\log_{10} [\text{Na}^+]) + 0.41(\%G+C) - 600/1,$$

where 1 is the length of the hybrids in nucleotides.  $T_m$  decreases approximately  $1^{\circ}$ - $1.5^{\circ}$  C. with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions. Thus, as used herein:

- (1) HIGH STRINGENCY refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at  $65^{\circ}$  C. (i.e., if a hybrid is not stable in 0.018M NaCl at  $65^{\circ}$  C., it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5 $\times$  Denhardt's solution, 5 $\times$  SSPE, 0.2% SDS at  $42^{\circ}$  C., followed by washing in 0.1 $\times$  SSPE, and 0.1% SDS at  $65^{\circ}$  C.;
- (2) MODERATE STRINGENCY refers to conditions equivalent to hybridization in 50% formamide, 5 $\times$  Denhardt's solution, 5 $\times$  SSPE, 0.2% SDS at  $42^{\circ}$  C., followed by washing in 0.2 $\times$  SSPE, 0.2% SDS, at  $65^{\circ}$  C.; and
- (3) LOW STRINGENCY refers to conditions equivalent to hybridization in 10% formamide, 5 $\times$  Denhardt's solution, 6 $\times$  SSPE, 0.2% SDS, followed by washing in 1 $\times$  SSPE, 0.2% SDS, at  $50^{\circ}$  C.

It is understood that these conditions may be duplicated using a variety of buffers and temperatures and that they are not necessarily precise.

Denhardt's solution and SSPE (see, e.g., Sambrook, Fritsch, and Maniatis, in: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers. For example, SSPE is pH 7.4 phosphate-buffered 0.18M NaCl. SSPE can be prepared, for example, as a 20 $\times$  stock solution by dissolving 175.3 g of NaCl, 27.6 g of  $\text{NaH}_2\text{PO}_4$  and 7.4 g EDTA in 800 ml of water, adjusting the pH to 7.4, and then adding water to 1 liter. Denhardt's solution (see, Denhardt (1966) *Biochem. Biophys. Res. Commun.* 23:641) can be prepared, for example, as a 50 $\times$  stock solution by mixing 5 g Ficoll (Type 400, Pharmacia LKB Biotechnology, INC., Piscataway N.J.), 5 g of polyvinylpyrrolidone, 5 g bovine serum albumin (Fraction V; Sigma, St. Louis Mo.) water to 500 ml and filtering to remove particulate matter.

The phrase "substantial sequence homology" is used herein in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the amino acid sequence of protein, that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species having substantial sequence homology are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard,

"slight and non-consequential sequence variations" mean that "homologous" sequences, i.e., sequences that have substantial homology with the DNA, RNA, or proteins disclosed and claimed herein, are functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

In practice, the term substantially the same sequence means that DNA or RNA encoding two proteins hybridize under conditions of high stringency and encode proteins that have the same sequence of amino acids or have changes in sequence that do not alter their structure or function. As used herein, substantially identical sequences of nucleotides share at least about 90% identity, and substantially identical amino acid sequences share more than 95% amino acid identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

As used herein, " $\alpha_2$  subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ.ID.No:1, or to the DNA of deposited clone having ATCC Accession No. 68277, or to DNA that encodes the amino acid sequence set forth in SEQ.ID.No:2. Typically, unless an  $\alpha_2$  subunit arises as a splice variant, an  $\alpha_2$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_2$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA described herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, " $\alpha_3$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ.ID.No:3, or to the DNA of deposited clone having ATCC Accession No. 68278, or to DNA that encodes the amino acid sequence set forth in SEQ.ID.No:4. Typically, unless an  $\alpha_3$  arises as a splice variant, an  $\alpha_3$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_3$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above described DNA.

As used herein, " $\alpha_5$  subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, as described, for example, by Chini et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:1572-1576.

The phrase "substantially the same" is used herein in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the amino acid sequence of protein, that have

slight and non-consequential sequence variations from the actual sequences disclosed herein. Species that are substantially the same are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" mean that sequences that are substantially the same as the DNA, RNA, or proteins disclosed and claimed herein are functionally equivalent to the human-derived sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the human-derived nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode human-derived proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution on a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

As used herein, " $\alpha_4$  subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example

said DNA may encode the amino acid sequence set forth in SEQ.ID.No:6, or

said DNA may encode the amino acid sequence encoded by clone HnAChR $\alpha$ 4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA may encode the amino acid sequence encoded by clone HnAChR $\alpha$ 4.1, deposited under ATCC Accession No. 69152.

Presently preferred  $\alpha_4$ -encoding DNAs can be characterized as follows

said DNA may hybridize to the coding sequence set forth in SEQ.ID.No:5 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 184-2067) under high stringency conditions, or

said DNA may hybridize under high stringency conditions to the sequence (preferably to substantially the entire sequence) of the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha$ 4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA may hybridize under high stringency conditions to the sequence of the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha$ 4.1, deposited under ATCC Accession No. 69152.

Especially preferred  $\alpha_4$ -encoding DNAs of the invention are characterized as follows

DNA having substantially the same nucleotide sequence as the coding region set forth in SEQ.ID.No:5 (i.e., nucleotides 184-2067 thereof), or

DNA having substantially the same nucleotide sequence as the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha$ 4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA have substantially the same sequence as the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha_4$ .1, deposited under ATCC Accession No. 69152.

Typically, unless an  $\alpha_4$  subunit arises as a splice variant,  $\alpha_4$ -encoding DNA will share substantial sequence homology (i.e., greater than about 90%), with the  $\alpha_4$  DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNAs.

As used herein, " $\alpha_3$  subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 7, or to DNA that encodes the amino acid sequence set forth in SEQ ID No. 8. Typically, unless an  $\alpha_5$  subunit arises as a splice variant, an  $\alpha_5$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_5$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA described herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA. Human  $\alpha_5$  subunit DNA has been described in the art, for example, by Chini et al. (1992) Proc. Natl. Acad. Sci. USA 89: 1572-1576.

As used herein, " $\alpha_6$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 9, or to DNA that encodes the amino acid sequence set forth in SEQ ID No. 10. Typically, unless an  $\alpha_6$  arises as a splice variant, an  $\alpha_6$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_6$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above described DNA.

As used herein, " $\alpha_7$  subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example, the nucleotides of said DNA may encode the amino acid sequence set forth in SEQ.ID.No:8. Presently preferred  $\alpha_7$ -encoding DNAs can be characterized as DNA which hybridizes under high stringency conditions to the coding sequence set forth in SEQ.ID.No:7 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 73-1581). Especially preferred  $\alpha_7$ -encoding DNAs of the invention are characterized as having substantially the same nucleotide sequence as the coding sequence set forth in SEQ.ID.No:7 (i.e., nucleotides 73-1581 thereof).

Typically, unless an  $\alpha_7$  subunit arises as a splice variant,  $\alpha_7$ -encoding DNA will share substantial sequence homology (greater than about 90%) with the  $\alpha_7$  DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such DNA would include regions of nearly 100% homology to the above-described DNA.



The  $\alpha_7$  subunits derived from the above-described DNA are expected to bind to the neurotoxin  $\alpha$ -bungarotoxin  $\alpha$ -bgtx). The activity of AChRs that contain  $\alpha_7$  subunits should be inhibited upon interaction with  $\alpha$ -bgtx. Amino acid residues 210 through 217, as set forth in SEQ.ID.No:8, are believed to be important elements in the binding of  $\alpha$ -bgtx (see, for example, Chargeux et al. Trends Pharmacol Sci. (1992) 13:299-301).

As used herein, a human beta subunit gene is a gene that encodes a beta subunit of a human neuronal nicotinic acetylcholine receptor. Assignment of the name "beta" to a putative nNACHR subunit, according to Deneris et al. supra, is based on the lack of adjacent cysteine residues (which are characteristic of alpha subunits). The beta subunit is frequently referred to as the structural NACHR subunit (although it is possible that beta subunits also have ACh binding properties). Combination of beta subunit(s) with appropriate alpha subunit(s) leads to the formation of a functional receptor. As used herein, a beta subunit subtype refers to a nNACHR subunit that is encoded by DNA that hybridizes under high stringency conditions to at least one of the nNACHR-encoding DNAs (or deposited clones) disclosed herein. A beta subunit forms a functional NACHR, as assessed by methods described herein or known to those of skill in this art, with appropriate alpha subunit subtype(s).

Also contemplated are beta subunits encoded by DNAs that encode beta subunits as defined above, but that by virtue of degeneracy of the genetic code do not necessarily hybridize to the disclosed DNA or deposited clones under the specified hybridization conditions. Such subunits also form functional receptors, as assessed by the methods described herein or known to those of skill in the art, in combination with appropriate alpha subunit subtype(s). Typically, unless a beta subunit is encoded by RNA that arises as a splice variant, beta-encoding DNA and the beta subunit encoded thereby share substantial sequence homology with the beta-encoding DNA and beta subunit protein described herein. It is understood that DNA or RNA encoding a splice variant may share less than 90% overall homology with the DNA or RNA provided herein, but such DNA will include regions of nearly 100% homology to the DNA described herein.

As used herein, " $\beta_2$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 13, or to the DNA of deposited clone HnAChR $\beta_2$ , having ATCC Accession No. 68279, or to DNA encoding the amino acid sequence set forth in SEQ ID No. 14. Typically, unless a  $\beta_2$  subunit arises as a splice variant, a  $\beta_2$  DNA shares substantial sequence homology (greater than about 90%) with the  $\beta_2$  DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, " $\beta_3$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 15, or to DNA encoding the amino acid sequence set forth in SEQ ID No. 16. Typically, unless a  $\beta_3$  subunit arises as a splice variant, a  $\beta_3$  DNA shares substantial sequence homology (greater than about 90%) with the  $\beta_3$  DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but

such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, " $\beta_4$  subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example, the nucleotides of said DNA may encode the amino acid sequence set forth in SEQ.ID.No:18. Presently preferred  $\beta_4$ -encoding DNAs can be characterized as DNA which hybridizes under high stringency conditions to the coding sequence set forth in SEQ.ID.No:17 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 87-1583). Especially preferred  $\beta_4$ -encoding DNAs of the invention are characterized as having substantially the same nucleotide sequence as set forth in SEQ.ID.No:17.

Typically, unless a  $\beta_4$  subunit arises as a splice variant,  $\beta_4$ -encoding DNA will share substantial sequence homology (greater than about 90%) with the  $\beta_4$  DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such DNA would include regions of nearly 100% homology to the above-described DNA.

DNA encoding human neuronal nicotinic AChR alpha and beta subunits may be isolated by screening suitable human cDNA or human genomic libraries under suitable hybridization conditions with DNA disclosed herein (including nucleotides derived from any of SEQ.ID.Nos:1, 3, 5, 7, 9, 11, 13, 15 or 17, or with any of the deposited clones referred to herein. Suitable libraries can be prepared from neuronal tissue samples, hippocampus tissue, or cell lines, such as the human neuroblastoma cell line IMR32 (ATCC Accession No. CCL127), and the like. The library is preferably screened with a portion of DNA including the entire subunit-encoding sequence thereof, or the library may be screened with a suitable probe.

As used herein, a probe is single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or the complement of) any 14 bases set forth in any of SEQ.ID.Nos:1, 3, 5, 7, 9, or 11, or in the subunit encoding DNA in any of the deposited clones described herein (e.g., ATCC accession no. 69239 or 69152). Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode transmembrane domains, sequences predicted to encode the cytoplasmic loop, signal sequences, acetylcholine (ACh) and  $\alpha$ -bungarotoxin ( $\alpha$ -bgtx) binding sites, and the like. Amino acids 210-220 are typically involved in ACh and  $\alpha$ -bgtx binding. The approximate amino acid residues which comprise such regions for other preferred probes are set forth in the following table:

Subunit	Signal Sequence	TMD1*	TMD2	TMD3	TMD4	Cytoplasmic Loop
$\alpha_2$	1-55	264-289	297-320	326-350	444-515	351-443
$\alpha_3$	1-30	240-265	273-296	302-326	459-480	327-458
$\alpha_4$	1-33	241-269	275-289	303-330	593-618	594-617

$\alpha_5$	1-22	250-275	282-304	310-335	422-437	336-421
$\alpha_6$	1-30	240-265	272-294	301-326	458-483	327-457
$\alpha_7$	1-23	229-256	262-284	290-317	462-487	318-461
$\beta_2$	1-25	234-259	267-288	295-320	453-477	321-452
$\beta_3$	1-20	232-258	265-287	293-318	421-446	319-420
$\beta_4$	1-23	234-258	264-285	290-319	454-478	320-453

\*TMD = transmembrane domain

Alternatively, portions of the DNA can be used as primers to amplify selected fragments in a particular library.

After screening the library, positive clones are identified by detecting a hybridization signal; the identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, by comparison with the sequences set forth herein or with the deposited clones described herein, to ascertain whether they include DNA encoding a complete alpha or beta subunit. If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If desired, the library can be rescreened with positive clones until overlapping clones that encode an entire alpha or beta subunit are obtained. If the library is a cDNA library, then the overlapping clones will include an open reading frame. If the library is genomic, then the overlapping clones may include exons and introns. In both instances, complete clones may be identified by comparison with the DNA and encoded proteins provided herein.

Complementary DNA clones encoding various subtypes of human nNACHR alpha and beta subunits have been isolated. Each subtype of the subunit appears to be encoded by a different gene. The DNA clones provided herein may be used to isolate genomic clones encoding each subtype and to isolate any splice variants by screening libraries prepared from different neural tissues. Nucleic acid amplification techniques, which are well known in the art, can be used to locate splice variants of human NACHR subunits. This is accomplished by employing oligonucleotides based on DNA sequences surrounding divergent sequence(s) as primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal the existence of splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns that correspond to different splice variants of transcripts encoding human NACHR subunits.

It has been found that not all subunit subtypes are expressed in all neural tissues or in all portions of the brain. Thus, in order to isolate cDNA encoding particular subunit subtypes or splice variants of such subtypes, it is preferable to screen libraries prepared from different neuronal or neural tissues. Preferred libraries for obtaining DNA encoding each subunit include: hippocampus to isolate human  $\alpha_4$ - and  $\alpha_5$ -encoding DNA; IMR32 to isolate human  $\alpha_3$ -,  $\alpha_5$ -,  $\alpha_7$ - and  $\beta_4$ -encoding DNA, thalamus to isolate  $\alpha_2$  and  $\beta_2$ -encoding DNA; and the like.

It appears that the distribution of expression of human neuronal nicotinic AChRs differs from the distribution of such receptors in rat. For example, RNA encoding the rat  $\alpha_4$  subunit is abundant

in rat thalamus, but is not abundant in rat hippocampus (see, e.g., Wada et al. (1989) *J. Comp. Neurol.* 284:314-335). No  $\alpha_4$ -encoding clones could be obtained, however, from a human thalamus library. Instead, human  $\alpha_4$  clones were ultimately obtained from a human hippocampus library. Thus, the distribution of  $\alpha_4$  nNACHR subunit in humans and rats appears to be quite different.

Rat  $\alpha_3$  subunit appears to be a CNS-associated subunit that is abundantly expressed in the thalamus and weakly expressed in the brain stem (see, e.g., Boulter et al. (1986) *Nature* 319:368-374; Boulter et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7763-7767; and Wada et al. (1989) *J. Comp. Neurol.* 284:314-335). In efforts to clone DNA encoding the human nicotinic AChR  $\alpha_3$  subunit, however, several human libraries, including a thalamus library, were unsuccessfully screened. Surprisingly, clones encoding human  $\alpha_3$  subunit were ultimately obtained from a brain stem library and from IMR32 cells that reportedly express few, if any, functional nicotinic acetylcholine receptors (see, e.g., Gotti et al. ((1986) *Biochem. Biophys. Res. Commun.* 137:1141-1147, and Clementi et al. (1986) *J. Neurochem.* 47:291-297).

Rat  $\alpha_7$  subunit transcript reportedly is abundantly expressed in the hippocampus (see Seguela et al. (1993) *J. Neurosci.* 13:596-604). Efforts to clone DNA encoding a human  $\alpha_7$  subunit from a human hippocampus library ( $1 \times 10^6$  recombinants) were unsuccessful. Surprisingly, clones encoding a human NACHR  $\alpha_7$  subunit were ultimately obtained from an IMR32 cell cDNA library.

The above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the level of skill of the art.

An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of affecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Presently preferred plasmids for expression of invention AChR subunits in eukaryotic host cells, particularly mammalian cells, include SV40 promoter-based expression vectors, such as pZeoSV (Invitrogen, San Diego, CA) CMV; cytomegalovirus (CMV) promoter-based vectors such as, pcDNA1, pcDNA3, pCEP4, (Invitrogen, San Diego, CA); and MMTV promoter-based vector such as pMAMneo (Clontech, Inc.) and the like.

As used herein, a promoter region refers to a segment of DNA that controls transcription of DNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors.

Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the like.

As used herein, the term "operatively linked" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to remove or alter 5' untranslated portions of the clones to remove extra, potential alternative translation initiation (i.e., start) codons or other sequences that interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, for example, Kozak (1991) J. Biol. Chem. 266:19867-19870) can be inserted immediately 5' of the start codon to enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, expression refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCDNA1 (Invitrogen, San Diego, Calif.), and MMTV promoter-based vectors such as pMSG (Catalog No. 27-4506-01 from Pharmacia, Piscataway, N.J.).

Full-length DNAs encoding human neuronal NACHR subunits have been inserted into vector pCMV-T7, a pUC19-based mammalian cell expression vector containing the CMV promoter/enhancer, SV40 splice/donor sites located immediately downstream of the promoter, a polylinker downstream of the splice/donor sites, followed by an SV40 polyadenylation signal. Placement of NACHR subunit DNA between the CMV promoter and SV40 polyadenylation signal provides for constitutive expression of the foreign DNA in a mammalian host cell transfected with the construct. For inducible expression of human NACHR subunit-encoding DNA in a mammalian cell, the DNA can be inserted into a plasmid such as PMSG. This plasmid contains the mouse mammary tumor virus (MMTV) promoter for steroid-inducible expression of operatively associated foreign DNA. If the host cell does not express endogenous glucocorticoid receptors required for uptake of glucocorticoids (i.e., inducers of the MMTV promoter) into the cell, it is necessary to additionally transfect the cell with DNA encoding the glucocorticoid receptor (ATCC accession no. 67200). Full-length human DNA clones encoding

human  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_7$ ,  $\beta_2$  and  $\beta_4$  have also been subcloned into pIBI24 (International Biotechnologies, Inc., New Haven, Conn.) or pCMV-T7-2 for synthesis of *in vitro* transcripts.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described polynucleic acids (i.e., DNA or mRNA). Such host cells as bacterial, yeast and mammalian cells can be used for replicating DNA and producing nAChR subunit(s). Methods for constructing expression vectors, preparing *in vitro* transcripts, transfecting DNA into mammalian cells, injecting oocytes, and performing electrophysiological and other analyses for assessing receptor expression and function as described herein are also described in PCT Application Nos. PCT/US91/02311, PCT/US91/05625 and PCT/US92/11090, and in co-pending U.S. application Ser. Nos. 07/504,455, 07/563,751 and 07/812,254. The subject matter of these applications are hereby incorporated by reference herein in their entirety.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press). Heterologous DNA may be introduced into host cells by any method known to those of skill in the art, such as transfection with a vector encoding the heterologous DNA by  $\text{CaPO}_4$  precipitation (see, e.g., Wigler et al. (1979) *Proc. Natl. Acad. Sci.* 76:1373-1376). Recombinant cells can then be cultured under conditions whereby the subunit(s) encoded by the DNA is (are) expressed. Preferred cells include mammalian cells (e.g., HEK 293, CHO and Ltk<sup>-</sup> cells), yeast cells (e.g., methylotrophic yeast cells, such as *Pichia pastoris*), bacterial cells (e.g., *Escherichia coli*), and the like.

While the DNA provided herein may be expressed in any eukaryotic cell, including yeast cells (such as, for example, *P. pastoris* (see U.S. Pat. Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), *Saccharomyces cerevisiae*, *Candida tropicalis*, *Hansenula polymorpha*, and the like), mammalian expression systems, including commercially available systems and other such systems known to those of skill in the art, for expression of DNA encoding the human neuronal nicotinic AChR subunits provided herein are presently preferred. *Xenopus* oocytes are preferred for expression of RNA transcripts of the DNA.

In preferred embodiments, DNA is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express a specific human nAChR receptor subtype, or specific combinations of subtypes. The resulting cell lines can then be produced in quantity for reproducible quantitative analysis of the effects of drugs on receptor function. In other embodiments, mRNA may be produced by *in vitro* transcription of DNA encoding each subunit. This mRNA, either from a single subunit clone or from a combination of clones, can then be injected into *Xenopus* oocytes where the RNA directs the synthesis of the human receptor subunits, which then form functional receptors. Alternatively, the subunit-encoding DNA can be directly injected into oocytes for expression of functional receptors. The transfected mammalian cells or injected oocytes may then be used in the methods of drug screening provided herein.

Cloned full-length DNA encoding any of the subunits of human neuronal nicotinic AChR may be introduced into a plasmid vector for expression in a eukaryotic cell. Such DNA may be

genomic DNA or cDNA. Host cells may be transfected with one or a combination of plasmids, each of which encodes at least one human neuronal nicotinic AChR subunit.

Eukaryotic cells in which DNA or RNA may be introduced include any cells that are transfectable by such DNA or RNA or into which such DNA or RNA may be injected. Preferred cells are those that can be transiently or stably transfected and also express the DNA and RNA. Presently most preferred cells are those that can form recombinant or heterologous human neuronal nicotinic AChRs comprising one or more subunits encoded by the heterologous DNA. Such cells may be identified empirically or selected from among those known to be readily transfected or injected.

Exemplary cells for introducing DNA include cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g., *Xenopus laevis* oocytes), yeast cells (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK 293 (which are available from ATCC under accession #CRL 1573; Ltk<sup>-</sup> cells (which are available from ATCC under accession #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhfr<sup>-</sup> CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12:555). Presently preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that have been adapted for growth in suspension and that can be frozen in liquid nitrogen and then thawed and regrown. HEK 293 cells are described, for example, in U.S. Pat. No. 5,024,939 to Gorman (see, also, Stillman et al. (1985) Mol. Cell. Biol. 5:2051-2060).

DNA may be stably incorporated into cells or may be transiently introduced using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene (such as, for example, the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance, and the like), and growing the transfected cells under conditions selective for cells expressing the marker gene. To produce such cells, the cells should be transfected with a sufficient concentration of subunit-encoding nucleic acids to form human neuronal nicotinic AChRs that contain the human subunits encoded by heterologous DNA. The precise amounts and ratios of DNA encoding the subunits may be empirically determined and optimized for a particular combination of subunits, cells and assay conditions. Recombinant cells that express neuronal nicotinic AChR containing subunits encoded only by the heterologous DNA or RNA are especially preferred.

Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing recombinant cells are known to the skilled artisan. Similarly, the human neuronal nicotinic AChR subunits may be purified using protein purification methods known to those of skill in the art. For example, antibodies or other ligands that specifically bind to one or more of the subunits may be used for affinity purification of the subunit or human neuronal nicotinic AChRs containing the subunits.

In accordance with one embodiment of the present invention, methods for producing cells that express human neuronal nicotinic AChR subunits and functional receptors are also provided. In one such method, host cells are transfected with DNA encoding at least one alpha subunit of a neuronal nicotinic acetylcholine receptor and at least one beta subunit of a neuronal nicotinic acetylcholine receptor. Using methods such as northern blot or slot blot analysis, transfected cells that contain alpha and/or beta subunit encoding DNA or RNA can be selected. Transfected cells are also analyzed to identify those that express NACHR protein. Analysis can be carried out, for example, by measuring the ability of cells to bind acetylcholine, nicotine, or a nicotine agonist, compared to the nicotine binding ability of untransfected host cells or other suitable control cells, by electrophysiologically monitoring the currents through the cell membrane in response to a nicotine agonist, and the like.

In particularly preferred aspects, eukaryotic cells which contain heterologous DNAs express such DNA and form recombinant functional neuronal nicotinic AChR(s). In more preferred aspects, recombinant neuronal nicotinic AChR activity is readily detectable because it is a type that is absent from the untransfected host cell or is of a magnitude not exhibited in the untransfected cell. Such cells that contain recombinant receptors could be prepared, for example, by causing cells transformed with DNA encoding the human neuronal nicotinic AChR  $\alpha_3$  and  $\beta_4$  subunits to express the corresponding proteins. The resulting synthetic or recombinant receptor would contain only the  $\alpha_3$  and  $\beta_4$  nNACHR subunits. Such a receptor would be useful for a variety of applications, e.g., as part of an assay system free of the interferences frequently present in prior art assay systems employing non-human receptors or human tissue preparations. Furthermore, testing of single receptor subunits with a variety of potential agonists or antagonists would provide additional information with respect to the function and activity of the individual subunits. Such information may lead to the identification of compounds which are capable of very specific interaction with one or more of the receptor subunits. Such specificity may prove of great value in medical application.

Thus, DNA encoding one or more human neuronal nicotinic AChR subunits may be introduced into suitable host cells (e.g., eukaryotic or prokaryotic cells) for expression of individual subunits and functional NACHRs. Preferably combinations of alpha and beta subunits may be introduced into cells: such combinations include combinations of any one or more of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$  and  $\alpha_7$  with  $\beta_2$  or  $\beta_4$ . Sequence information for  $\alpha_1$  is presented in Biochem. Soc. Trans. (1989) 17:219-220; sequence information for  $\alpha_5$  is presented in Proc. Natl. Acad. Sci. USA (1992) 89:1572-1576; and sequence information for  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_7$ ,  $\beta_2$  and  $\beta_4$  is presented in the Sequence Listing provided herewith. Presently preferred combinations of subunits include any one or more of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  with  $\beta_4$ ; or  $\alpha_4$  or  $\alpha_7$  in combination with either  $\beta_2$  or  $\beta_4$ . It is recognized that some of the subunits may have ion transport function in the absence of additional subunits. For example, the  $\alpha_7$  subunit is functional in the absence of any added beta subunit.

In accordance with the above, also disclosed are cells transfected or transformed with DNA or RNA encoding multimeric human NACHR subunit combinations. These include but are not limited to the following:

#### Multimeric Subunit Combinations



$\alpha 2\beta 4\alpha 6$   
 $\alpha 3\beta 4\alpha 6$   
 $\alpha 4\beta 4\alpha 5$   
 $\alpha 4\beta 4\alpha 6$   
 $\alpha 4\beta 2\alpha 5$   
 $\alpha 4\beta 2\beta 3$   
 $\alpha 3\beta 2\alpha 6\beta 3$   
 $\alpha 2\beta 4\alpha 5$   
 $\alpha 2\beta 2\alpha 5$   
 $\alpha 3\beta 2\alpha 5$   
 $\alpha 3\beta 4\alpha 5$

Also contemplated are cells expressing one or more  $\alpha$  subunit with more than one  $\beta$  subunit.

These include but are not limited to the following subunit combinations:

$\alpha X\beta 2\beta 4$  (where X defines one or more of the alpha subunits disclosed herein)

$\alpha X\beta 2\beta 3\beta 4$

$\alpha 2\beta 2\alpha 6$

$\alpha 3\beta 2\alpha 6$

$\alpha 4\beta 2\alpha 6$

$\alpha X\beta 2\beta 3$  (where X defines one or more of the alpha subunits disclosed herein)

Stable cell lines expressing any of the above referenced multimeric subunit combinations are also a feature of the invention.

As used herein, " $\beta_2$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ.ID.No:9, or to the DNA of deposited clone HnACh $\beta$ 62, having ATCC Accession No. 68279, or to DNA encoding the amino acid sequence set forth in SEQ.ID.No:10. Typically, unless a  $\beta_2$  subunit arises as a splice variant, a  $\beta_2$  DNA shares substantial sequence homology (greater than about 90%) with the  $\beta_2$  DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

In certain embodiments, eukaryotic cells with heterologous human neuronal nicotinic AChRs are produced by introducing into the cell a first composition, which contains at least one RNA transcript that is translated in the cell into a subunit of a human neuronal nicotinic AChR. In preferred embodiments, the subunits that are translated include an alpha subunit of a human neuronal nicotinic AChR. More preferably, the composition that is introduced contains an RNA transcript which encodes an alpha subunit and also contains an RNA transcript which encodes a beta subunit of a human neuronal nicotinic AChR. RNA transcripts can be obtained from cells transfected with DNAs encoding human neuronal nicotinic acetylcholine receptor subunits or by in vitro transcription of subunit-encoding DNAs. Methods for in vitro transcription of cloned DNA and injection of the resulting mRNA into eukaryotic cells are well known in the art.

Amphibian oocytes are particularly preferred for expression of in vitro transcripts of the human nAChR DNA clones provided herein. See, for example, Dascal (1989) CRC Crit. Rev. Biochem. 22:317-387, for a review of the use of *Xenopus* oocytes to study ion channels.

Thus, pairwise (or stepwise) introduction of DNA or RNA encoding alpha and beta subtypes into cells is possible. The resulting cells may be tested by the methods provided herein or known to those of skill in the art to detect functional AChR activity. Such testing will allow the identification of pairs of alpha and beta subunit subtypes that produce functional AChRs, as well as individual subunits that produce functional AChRs.

An alternative embodiment is drawn to a non-human cell line that stably expresses the  $\alpha_7$  nAChR. Preferably, the non-human cell line expressing the human  $\alpha_7$  nAChR subunit is a rat cell line, i.e., the GH<sub>4</sub>C<sub>1</sub> cell line.

As used herein, GH<sub>4</sub>C<sub>1</sub> cells are derived from rat pituitary tumor tissue and are transfected with DNA or RNA encoding the human  $\alpha_7$  nAChR.

As used herein, activity of a human neuronal nicotinic AChR refers to any activity characteristic of an nAChR. Such activity can typically be measured by one or more in vitro methods, and frequently corresponds to an in vivo activity of a human neuronal nicotinic AChR. Such activity may be measured by any method known to those of skill in the art, such as, for example, measuring the amount of current which flows through the recombinant channel in response to a stimulus.

Methods to determine the presence and/or activity of human neuronal nicotinic AChRs include assays that measure nicotine binding, <sup>86</sup>Rb ion-flux, Ca<sup>2+</sup> influx, the electrophysiological response of cells, the electrophysiological response of oocytes transfected with RNA from the cells, and the like. In particular, methods are provided herein for the measurement or detection of an AChR-mediated response upon contact of cells containing the DNA or mRNA with a test compound.

As used herein, a recombinant or heterologous human neuronal nicotinic AChR refers to a receptor that contains one or more subunits encoded by heterologous DNA that has been introduced into and expressed in cells capable of expressing receptor protein. A recombinant human neuronal nicotinic AChR may also include subunits that are produced by DNA endogenous to the host cell. In certain embodiments, recombinant or heterologous human neuronal nicotinic AChR may contain only subunits that are encoded by heterologous DNA.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome of the cell in which it is present or to DNA or RNA which is found in a location or locations in the genome that differ from that in which it occurs in nature. Typically, heterologous or foreign DNA and RNA refers to DNA or RNA that is not endogenous to the host cell and has been artificially introduced into the cell. Examples of heterologous DNA include DNA that encodes a human neuronal nicotinic AChR subunit, DNA that encodes RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes, and the

like. The cell that expresses heterologous DNA may contain DNA encoding the same or different expression products. Heterologous DNA need not be expressed and may be integrated into the host cell genome or maintained episomally.

Recombinant receptors on recombinant eukaryotic cell surfaces may contain one or more subunits encoded by the DNA or mRNA encoding human neuronal nicotinic AChR subunits, or may contain a mixture of subunits encoded by the host cell and subunits encoded by heterologous DNA or mRNA. Recombinant receptors may be homogeneous or may be a mixture of subtypes. Mixtures of DNA or mRNA encoding receptors from various species, such as rats and humans, may also be introduced into the cells. Thus, a cell can be prepared that expresses recombinant receptors containing only  $\alpha_3$  and  $\beta_4$  subunits, or any other combination of alpha and beta subunits provided herein. For example,  $\alpha_4$  and/or  $\alpha_7$  subunits of the present invention can be co-expressed with  $\beta_2$  and/or  $\beta_4$  receptor subunits; similarly,  $\beta_4$  subunits according to the present invention can be co-expressed with  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$  and/or  $\alpha_7$  receptor subunits. As noted previously, some of the nNACHR subunits may be capable of forming functional receptors in the absence of other subunits, thus co-expression is not always required to produce functional receptors.

As used herein, a functional neuronal nicotinic AChR is a receptor that exhibits an activity of neuronal nicotinic AChRs as assessed by any in vitro or in vivo assay disclosed herein or known to those of skill in the art. Possession of any such activity that may be assessed by any method known to those of skill in the art and provided herein is sufficient to designate a receptor as functional. Methods for detecting NACHR protein and/or activity include, for example, assays that measure nicotine binding,  $^{86}\text{Rb}$  ion-flux,  $\text{Ca}^{2+}$  influx, the electrophysiological response of cells containing heterologous DNA or mRNA encoding one or more receptor subunit subtypes, and the like. Since all combinations of alpha and beta subunits may not form functional receptors, numerous combinations of alpha and beta subunits should be tested in order to fully characterize a particular subunit and cells which produce same. Thus, as used herein, "functional" with respect to a recombinant or heterologous human neuronal nicotinic AChR means that the receptor channel is able to provide for and regulate entry of human neuronal nicotinic AChR-permeable ions, such as, for example,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ , in response to a stimulus and/or bind ligands with affinity for the receptor. Preferably such human neuronal nicotinic AChR activity is distinguishable, such as by electrophysiological, pharmacological and other means known to those of skill in the art, from any endogenous nicotinic AChR activity that may be produced by the host cell.

In accordance with a particular embodiment of the present invention, recombinant human neuronal nicotinic AChR-expressing mammalian cells or oocytes can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the AChR-mediated response in the presence and absence of test compound, or by comparing the AChR-mediated response of test cells, or control cells (i.e., cells that do not express nNACHRs), to the presence of the compound.

As used herein, a compound or signal that "modulates the activity of a neuronal nicotinic AChR" refers to a compound or signal that alters the activity of NACHR so that activity of the NACHR is different in the presence of the compound or signal than in the absence of the compound or

signal. In particular, such compounds or signals include agonists and antagonists. The term agonist refers to a substance or signal, such as ACh, that activates receptor function; and the term antagonist refers to a substance that interferes with receptor function. Typically, the effect of an antagonist is observed as a blocking of activation by an agonist. Antagonists include competitive and non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site specific for the agonist (e.g., ligand or neurotransmitter) for the same or closely situated site. A non-competitive antagonist or blocker inactivates the functioning of the receptor by interacting with a site other than the site that interacts with the agonist.

As understood by those of skill in the art, assay methods for identifying compounds that modulate human neuronal nicotinic AChR activity (e.g., agonists and antagonists) generally require comparison to a control. One type of a "control" cell or "control" culture is a cell or culture that is treated substantially the same as the cell or culture exposed to the test compound, except the control culture is not exposed to test compound. For example, in methods that use voltage clamp electrophysiological procedures, the same cell can be tested in the presence and absence of test compound, by merely changing the external solution bathing the cell. Another type of "control" cell or "control" culture may be a cell or a culture of cells which are identical to the transfected cells, except the cells employed for the control culture do not express functional human neuronal nicotinic AChRs. In this situation, the response of test cell to test compound is compared to the response (or lack of response) of receptor-negative (control) cell to test compound, when cells or cultures of each type of cell are exposed to substantially the same reaction conditions in the presence of compound being assayed.

The functional recombinant human neuronal nicotinic AChR includes at least an alpha subunit, or an alpha subunit and a beta subunit of a human neuronal nicotinic AChR. Eukaryotic cells expressing these subunits have been prepared by injection of RNA transcripts and by transfection of DNA. Such cells have exhibited nicotinic AChR activity attributable to human neuronal nicotinic AChRs that contain one or more of the heterologous human neuronal nicotinic AChR subunits. For example, *Xenopus laevis* oocytes that had been injected with in vitro transcripts of the DNA encoding human neuronal nicotinic AChR  $\alpha_3$  and  $\beta_4$  subunits exhibited AChR agonist induced currents; whereas cells that had been injected with transcripts of either the  $\alpha_3$  or  $\beta_4$  subunit alone did not. In addition, HEK 293 cells that had been co-transfected with DNA encoding human neuronal NACHR  $\alpha_3$  and  $\beta_4$  subunits exhibited AChR agonist-induced increases in intracellular calcium concentration, whereas control HEK 293 cells (i.e., cells that had not been transfected with  $\alpha_3$ - and  $\beta_4$ -encoding DNA) did not exhibit any AChR agonist-induced increases in intracellular calcium concentration.

With respect to measurement of the activity of functional heterologous human neuronal nicotinic AChRs, endogenous AChR activity and, if desired, activity of AChRs that contain a mixture of endogenous host cell subunits and heterologous subunits, should, if possible, be inhibited to a significant extent by chemical, pharmacological and electrophysiological means.

## Deposits

The deposited clones have been deposited at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md., U.S.A. 20852, under the terms of the Budapest Treaty on

the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted. In particular, upon issuance of a U.S. Patent based on this or any application claiming priority to or incorporating this application by reference thereto, all restrictions upon availability of the deposited material will be irrevocably removed.

The invention will now be described in greater detail with reference to the following non-limiting examples.

## EXAMPLE 1

### Isolation of DNA Encoding Human nNACHR Subunits

#### A. DNA Encoding a Human nNACHR $\beta_4$ Subunit

Random primers were used in synthesizing cDNA from RNA isolated from the IMR32 human neuroblastoma cell line (the cells had been treated with dibutyryl cAMP and bromodeoxyuridine prior to constructing the library). The library constructed from the cDNAs was screened with a fragment of a rat nicotinic AChR  $\beta_4$  subunit cDNA. Hybridization was performed at 42° C. in 5× SSPE, 5× Denhardt's solution, 50% formamide, 200  $\mu$ g/ml herring sperm DNA and 0.2% SDS. Washes were performed in 0.1× SSPE, 0.2% SDS at 65° C. Five clones were identified that hybridized to the probe.

The five clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. The insert DNA of one of the five clones contained the complete coding sequence of a  $\beta_4$  subunit of a human nicotinic AChR (see nucleotides 87-1583 of SEQ.ID.No:11). The amino acid sequence deduced from the nucleotide sequence of the full-length clone has ~81% identity with the amino acid sequence deduced from the rat nicotinic AChR  $\beta_4$  subunit DNA. Several regions of the deduced rat and human  $\beta_4$  amino acid sequences are notably dissimilar: amino acids 1-23 (the human sequence has only ~36% identity with respect to the rat sequence), 352-416 (the human sequence has only ~48% identity with respect to the rat sequence), and 417-492 (the human sequence has only ~78% identity with respect to the rat sequence). Furthermore, amino acids 376-379 in the rat  $\beta_4$  subunit are not contained in the human  $\beta_4$  subunit.

#### B. DNA Encoding a Human nNACHR $\alpha_7$ Subunit

An amplified IMR32 cell cDNA library ( $1 \times 10^6$  recombinants; cells treated with dibutyryl cAMP and bromodeoxyuridine) was screened with a fragment of a rat nicotinic AChR  $\alpha_7$  subunit cDNA. The hybridization conditions were identical to those described above for screening an IMR32 cell cDNA library with the rat  $\beta_4$  subunit DNA. Washes were performed in 0.2× SSPE,

0.2% SDS at 65° C. Seven positive clones were identified by hybridization to the labeled rat DNA probe. Six of the clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. One of the clones contains the complete coding sequence of a human AChR receptor  $\alpha_7$  subunit gene (see nucleotides 73-1581 of SEQ.ID.No:7).

#### C. DNA Encoding a Human nNACHR $\alpha_4$ Subunit

Random primers were used in synthesizing cDNA from RNA isolated from human hippocampus tissue. cDNAs larger than 2.0 kb were inserted into the  $\lambda$ gt10 phage vector to create a cDNA library. Approximately  $1 \times 10^6$  recombinants were screened with a fragment of a DNA encoding a rat nicotinic AChR  $\alpha_4$  subunit using the same hybridization and washing conditions as described above for screening an IMR32 cell cDNA library for  $\alpha_7$  subunit cDNAs. Three clones hybridized strongly to the probe. Two of these three clones, designated KE $\alpha$ 4.1 and KE $\alpha$ 4.2, have been deposited with the American Type Culture Collection (ATCC, Rockville, Md.) and assigned accession nos. 69152 and 69239, respectively.

Characterization of the plaque-purified clones revealed that one of the clones, KE $\alpha$ 4.2, contains the complete coding sequence of a human nicotinic AChR  $\alpha_4$  subunit gene (coding sequence of this human  $\alpha_4$  subunit cDNA is provided as nucleotides 184-2067 in SEQ.ID.No:5). Comparison of the 5' ends of the coding sequences of the human and rat  $\alpha_4$  subunit cDNAs reveals that the rat sequence contains an 18-nucleotide segment that is not present in the human sequence.

#### D. DNA Encoding Human nNACHR $\alpha_2$ , $\alpha_3$ , & $\beta_2$ Subunits

Plasmids containing DNA that encodes and/or that can be used to isolate DNA that encodes human neuronal nicotinic acetylcholine receptor  $\alpha_2$ ,  $\alpha_3$  and  $\beta_2$  subunits have been deposited with the American Type Culture Collection (ATCC). The clone names and deposit accession numbers are:

Subunit	Clone Name	ATCC Accession No.
$\alpha_2$	HnAChR $\alpha_2$	68277
$\alpha_3$	HnACHR $\alpha_3$	68278
$\beta_2$	HnAChR $\beta_2$	68279

In addition, DNA sequences that encode full-length  $\alpha_2$ ,  $\alpha_3$  and  $\beta_2$  subunits are set forth in SEQ.ID.Nos:1, 3 and 9, respectively.

#### EXAMPLE 2

## I. Preparation of Constructs for the Expression of Recombinant Human Neuronal Nicotinic AChR Subunits

Isolated cDNAs encoding human neuronal nicotinic AChR subunits were incorporated into vectors for use in expressing the subunits in mammalian host cells and for use in generating in vitro transcripts of the DNAs to be expressed in *Xenopus* oocytes. Several different vectors were utilized in preparing the constructs as follows.

### A. Construct for Expression of a Human nNACHR $\alpha_3$ Subunit

DNA encoding a human neuronal nicotinic AChR  $\alpha_3$  subunit was subcloned into the pCMV-T7-2 general expression vector to create pCMV-KE $\alpha_3$ . Plasmid pCMV-T7-2 (see FIG. 1) is a pUC19-based vector that contains a CMV promoter/enhancer, SV40 splice donor/splice acceptor sites located immediately downstream of the promoter, a T7 bacteriophage RNA polymerase promoter positioned downstream of the SV40 splice sites, an SV40 polyadenylation signal downstream of the T7 promoter, and a polylinker between the T7 promoter and the polyadenylation signal. This vector thus contains all the regulatory elements required for expression of heterologous DNA in a mammalian host cell, wherein the heterologous DNA has been incorporated into the vector at the polylinker. In addition, because the T7 promoter is located just upstream of the polylinker, this plasmid can be used for synthesis of in vitro transcripts of heterologous DNA that has been subcloned into the vector at the polylinker. FIG. 1 also shows a restriction map of pCMV-T7-3. This plasmid is identical to pCMV-T7-2 except that the restriction sites in the polylinker are in the opposite order as compared to the order in which they occur in pCMV-T7-2.

A 1.7 kb *Sfi*I (blunt-ended)/*Eco*RI DNA fragment containing nucleotides 27-1759 of SEQ.ID.No:3 (i.e., the entire  $\alpha_3$  subunit coding sequence plus 12 nucleotides of 5' untranslated sequence and 206 nucleotides of 3' untranslated sequence) was ligated to *Eco*RV/*Eco*RI-digested pCMV-T7-2 to generate pCMV-KE $\alpha_3$ . Plasmid pCMV-KE $\alpha_3$  was used for expression of the  $\alpha_3$  subunit in mammalian cells and for generating in vitro transcripts of the  $\alpha_3$  subunit DNA.

### B. Constructs for Expression of a Human nNACHR $\beta_4$ Subunit

A 1.9 kb *Eco*RI DNA fragment containing nucleotides 1-1915 of SEQ. ID.No:11 (i.e., the entire  $\beta_4$  subunit coding sequence plus 86 nucleotides of 5' untranslated sequence and 332 nucleotides of 3' untranslated sequence) was ligated to *Eco*RI-digested pGEM7Zf(+) (Promega Catalog #P2251; Madison, Wis.). The resulting construct, KE $\beta_4$ .6/pGEM, contains the T7 bacteriophage RNA polymerase promoter in operative association with two tandem  $\beta_4$  subunit DNA inserts (in the same orientation) and was used in generating in vitro transcripts of the DNA.

The same 1.9 kb *Eco*RI DNA fragment containing nucleotides 1-1915 of SEQ.ID.No:11 was ligated as a single insert to *Eco*RI-digested pCMV-T7-3 to generate pCMV-KE $\beta_4$ . Plasmid pCMV-KE $\beta_4$  was used for expression of the  $\beta_4$  subunit in mammalian cells and for generating in vitro transcripts of the  $\beta_4$  subunit DNA.

### C. Constructs for Expression of a Human nNACHR $\alpha_7$ Subunit

Two pCMV-T7-2-based constructs were prepared for use in recombinant expression of a human neuronal nicotinic AChR  $\alpha_7$  subunit. The first construct, pCMV-KE $\alpha_7$ .3, was prepared by ligating a 1.9 kb XhoI DNA fragment containing nucleotides 1-1876 of SEQ. ID.No:7 (i.e., the entire  $\alpha_7$  subunit coding sequence plus 72 nucleotides of 5' untranslated sequence and 295 nucleotides of 3' untranslated sequence) to SalI-digested pCMV-T7-3. The second construct, pCMV-KE $\alpha_7$ , was prepared by replacing the 5' untranslated sequence of the 1.9 kb XhoI  $\alpha_7$  subunit DNA fragment described above with a consensus ribosome binding site (5'-GCCACC-3'; see Kozak (1987) Nucl. Acids Res. 15:8125-8148). The resulting modified fragment was ligated as a 1.8 kb BglII/XhoI fragment with BglII/SalI-digested pCMV-T7-2 to generate pCMV-KE $\alpha_7$ . Thus, in pCMV-KE $\alpha_7$ , the translation initiation codon of the coding sequence of the  $\alpha_7$  subunit cDNA is preceded immediately by a consensus ribosome binding site.

#### D. Constructs for Expression of a Human nNAChR $\beta_2$ Subunit

DNA fragments encoding portions of a human neuronal nicotinic AChR  $\beta_2$  subunit were ligated together to generate a full-length  $\beta_2$  subunit coding sequence contained in plasmid pIBI24 (International Biotechnologies, Inc. (IBI), New Haven, Conn.). The resulting construct, H $\beta$ 2.1F, contains nucleotides 1-2450 of SEQ.ID.No:9 (i.e., the entire  $\beta_2$  subunit coding sequence, plus 266 nucleotides of 5' untranslated sequence and 675 nucleotides of 3' untranslated sequence) in operative association with the T7 promoter. Therefore, H $\beta$ 2.1F was used for synthesis of in vitro transcripts from the  $\beta_2$  subunit DNA..

Since the 5' untranslated sequence of the  $\beta_2$  subunit DNA contains a potential alternative translation initiation codon (ATG) beginning 11 nucleotides upstream (nucleotides 256-258 in SEQ.ID.No:9) of the correct translation initiation codon (nucleotides 267-269 in SEQ. ID.No:9), and because the use of the upstream ATG sequence to initiate translation of the  $\beta_2$  DNA would result in the generation of an inoperative peptide (because the upstream ATG is not in the correct reading frame), an additional  $\beta_2$ -encoding construct was prepared as follows. A 2.2 kb KspI/EcoRI DNA fragment containing nucleotides 262-2450 of SEQ.ID.No:9 was ligated to pCMV-T7-2 in operative association with the T7 promoter of the plasmid to create pCMV-KE $\beta_2$ . The  $\beta_2$  subunit DNA contained in pCMV-KE $\beta_2$  retains only 5 nucleotides of 5' untranslated sequence upstream of the correct translation initiation codon.

### EXAMPLE 3

#### Expression of Recombinant Human Nicotinic AChR in Oocytes

Xenopus oocytes were injected with in vitro transcripts prepared from constructs containing DNA encoding  $\alpha_3$ ,  $\alpha_7$ ,  $\beta_2$  and  $\beta_4$  subunits. Electrophysiological measurements of the oocyte transmembrane currents were made using the two-electrode voltage clamp technique (see, e.g., Stuhmer (1992) *Meth. Enzymol.* 207:319-339).

##### 1. Preparation of *in vitro* transcripts



Recombinant capped transcripts of pCMV-KE $\alpha$ 3, pCMV-KE $\beta$ 2, KE $\beta$ 4.6/pGEM and pCMV-KE $\beta$ 4 were synthesized from linearized plasmids using the mCAP RNA Capping Kit (Cat. #200350 from Stratagene, Inc., La Jolla, Calif.). Recombinant capped transcripts of pCMV-KE $\alpha$ 7, pCMV-KE $\alpha$ 7.3 and H $\beta$ 2.1F were synthesized from linearized plasmids using the MEGAscript T7 in vitro transcription kit according to the capped transcript protocol provided by the manufacturer (Catalog #1334 from AMBION, Inc., Austin, Tex.). The mass of each synthesized transcript was determined by UV absorbance and the integrity of each transcript was determined by electrophoresis through an agarose gel.

## 2. Electrophysiology

Xenopus oocytes were injected with either 12.5, 50 or 125 ng of human nicotinic AChR subunit transcript per oocyte. The preparation and injection of oocytes were carried out as described by Dascal (1987) in *Crit. Rev. Biochem.* 22:317-387. Two-to-six days following mRNA injection, the oocytes were examined using the two-electrode voltage clamp technique. The cells were bathed in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.3) containing 1  $\mu$ M atropine with or without 100  $\mu$ M d-tubocurarine. Cells were voltage-clamped at -60 to -80 mV. Data were acquired with Axotape software at 2-5 Hz. The agonists acetylcholine (ACh), nicotine, and cytisine were added at concentrations ranging from 0.1  $\mu$ M to 100  $\mu$ M. The results of electrophysiological analyses of the oocytes are summarized in Table 1.

TABLE 1

Template, ng RNA Injected	Number of oocytes responding	Current Agonists	Amplitude
pCMV-KE $\alpha$ 3, 12.5 ng	0 of 8	ACh, Nicotine	
KE $\beta$ 4.6/pGEM, 12.5 ng	0 of 9	ACh, Nicotine	
pCMV-KE $\alpha$ 3, 12.5 ng +	4 of 5	ACh, Nicotine	20-550 nA
KE $\beta$ 4.6/pGEM, 12.5 ng pCMV-KE $\alpha$ 3, 12.5 ng +	3 of 4	ACh, Cytisine, Nicotine	20-300 nA
KE $\beta$ 4.6/pGEM, 12.5 ng pCMV-KE $\alpha$ 3, 125 ng +	5 of 5	Ch, Nicotine, Cytisine	200-500 nA
and pCMV-KE $\beta$ 4, 125 ng pCMV-KE $\alpha$ 3, 125 ng +	6 of 6	ACh, Nicotine, Cytisine	100-400 nA
pCMV-KE $\beta$ 4, 125 ng pCMV-KE $\alpha$ 7.3, 125 ng	3 of 15	Ach	~20 nA
pCMV-KE $\alpha$ 7, 125 ng	11 of 11	Ach	20-250 nA
pCMV-KE $\alpha$ 3, 12.5 ng +	2 of 9	ACh, Nicotine	<10 nA
pCMV-KE $\beta$ 2, 12.5 ng pCMV-KE $\alpha$ 3, 125 ng +	0 of 9	ACh, Nicotine	
pCMV-KE $\beta$ 2, 125 ng pCMV-KE $\alpha$ 3, 125 ng +	13 of 16	Ach (100 $\mu$ M) ACh (300 $\mu$ M)	~20 nA ~80 nA
H $\beta$ 2.1 F, 125 ng			

#### a. Oocytes Injected with $\alpha_3$ and/or $\beta_4$ Transcripts

Oocytes that had been injected with 12.5 ng of the  $\alpha_3$  transcript or 12.5 ng of the  $\beta_4$  transcript did not respond to application of up to 100  $\mu\text{M}$  ACh, nicotine or cytosine. Thus, it appears that these subunits do not form functional homomeric nicotinic AChR channels. By contrast, oocytes injected with 12.5 or 125 ng of the  $\alpha_3$  transcript and 12.5 ng or 125 ng of the  $\beta_4$  transcript exhibited detectable inward currents in response to ACh, nicotine, and cytosine at the tested concentrations (0.1  $\mu\text{M}$  to 10  $\mu\text{M}$ ). Some differences in the kinetics of the responses to cytosine compared to nicotine and ACh were observed. The relative potency of the agonists appeared to be cytosine>ACh>nicotine, which differs from the results of similar studies of oocytes injected with transcripts of the rat nicotinic AChR  $\alpha_3$  and  $\beta_4$  subunits (see, for example, Luetje et al. (1991) *J. Neurosci.* 11:837-845).

The responses to ACh and nicotine were reproducibly blocked by d-tubocurarine. For example, complete blockage of the response to ACh was observed in the presence of 100  $\mu\text{M}$  d-tubocurarine. The inhibition appeared to be reversible. The responses to ACh, nicotine and cytosine were also at least partially blocked by 100 nM mecamylamine.

The current response of  $\alpha_3$ - $\beta_4$ -injected oocytes to 10  $\mu\text{M}$  ACh was also examined in terms of membrane voltage. In these experiments, voltage steps were applied to the cells in the presence of ACh. The graph of current vs. voltage appeared typical of responses observed for  $\text{Na}^+$ ,  $\text{K}^+$  - permeable channels. For example, the zero current level (reversal potential) is less than -40 mV. The contribution of  $\text{Ca}^{++}$  flux to the total current can be ascertained by varying the calcium concentration in the external medium and taking multiple current measurements at different holding potentials around the reversal potential. Such studies indicate that the channel carrying the current generated in response to ACh treatment of  $\alpha_3$ - $\beta_4$ -injected oocytes is permeable to  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$ .

#### b. Oocytes injected with $\alpha_7$ subunit transcripts

As described in Example 2, two constructs were prepared for use in expressing the human neuronal nicotinic AChR  $\alpha_7$  subunit. Plasmid pCMV-KE $\alpha_7$ .3 contains the  $\alpha_7$  subunit coding sequence with 72 nucleotides of 5' untranslated sequence upstream of the translation initiation codon. Plasmid pCMV-KE $\alpha_7$  contains the  $\alpha_7$  subunit coding sequence devoid of any 5' untranslated sequence and further contains a consensus ribosome binding site immediately upstream of the coding sequence.

Oocytes injected with 125 ng of  $\alpha_7$  transcript synthesized from pCMV-KE $\alpha_7$  displayed inward currents in response to 10 or 100  $\mu\text{M}$  ACh. This response was blocked by 100  $\mu\text{M}$  d-tubocurarine.

Oocytes injected with 125 ng of  $\alpha_7$  transcript synthesized from pCMV-KE $\alpha_7$ .3 exhibited ACh-induced currents that were substantially weaker than those of oocytes injected with  $\alpha_7$  transcript synthesized from pCMV-KE $\alpha_7$ . These results indicate that human neuronal nicotinic AChR  $\alpha_7$

subunit transcripts generated from  $\alpha_7$  subunit DNA containing a ribosome binding site in place of 5' untranslated sequence may be preferable for expression of the  $\alpha_7$  receptor in oocytes.

#### c. Oocytes injected with $\alpha_3$ and $\beta_2$ subunit transcripts

As described in Example 2, two constructs were prepared for use in expressing the human neuronal nicotinic AChR  $\beta_2$  subunit. Plasmid H $\beta_2$ .1F contains the  $\beta_2$  subunit coding sequence with 266 nucleotides of 5' untranslated sequence upstream of the translation initiation codon. Plasmid pCMV-KE $\beta_2$  contains the  $\beta_2$  subunit coding sequence and only 5 nucleotides of 5' untranslated sequence upstream of the translation initiation codon.

Oocytes injected with transcripts of pCMV-KE $\alpha_3$  and pCMV-KE $\beta_2$  displayed no current in response to nicotinic AChR  $\alpha_3$  agonists. In contrast, oocytes injected with transcripts of pCMV-KE $\alpha_3$  and H $\beta_2$ .1F displayed ~20 nA inward currents in response to 100  $\mu$ M ACh and ~80 nA inward currents in response to 300  $\mu$ M ACh. The current response was blocked by 100  $\mu$ M d-tubocurarine. These results indicate that human neuronal nicotinic AChR  $\beta_2$  subunit transcripts generated from  $\beta_2$  subunit DNA containing 5' untranslated sequence may be preferable to transcripts generated from  $\beta_2$  DNA containing only a small portion of 5' untranslated sequence for expression of the  $\alpha_3\beta_2$  receptors in oocytes.

### EXAMPLE 4

#### Recombinant Expression of Human nAChR Subunits in Mammalian Cells

##### I. Recombinant expression of human NACHR $\alpha_3$ and $\beta_4$ or $\alpha_7$ subunits in HEK 293 Cells:

Human embryonic kidney (HEK) 293 cells were transiently and stably transfected with DNA encoding human neuronal nicotinic AChR  $\alpha_3$  and  $\beta_4$ , or  $\alpha_7$  subunits. Transient transfectants were analyzed for expression of nicotinic AChR using various assays, e.g., electrophysiological methods,  $\text{Ca}^{2+}$ -sensitive fluorescent indicator-based assays and [ $^{125}$ I]- $\alpha$ -bungarotoxin-binding assays.

##### 1. Transient Transfection of HEK Cells

Two transient transfection were performed. In one transfection, HEK cells were transiently co-transfected with DNA encoding  $\alpha_3$  (plasmid pCMV-KE $\alpha_3$ ) and  $\beta_4$  (plasmid pCMV-KF $\beta_4$ ) subunits. In the other transfection, HEK cells were transiently transfected with DNA encoding the  $\alpha_7$  subunit (plasmid pCMV-KE $\alpha_7$ ). In both transfections,  $\sim 2 \times 10^6$  HEK cells were transiently transfected with 18  $\mu$ g of the indicated plasmid(s) according to standard  $\text{CaPO}_4$  transfection procedures [Wigler et al. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:1373-1376]. In addition, 2  $\mu$ g of plasmid pCMV $\beta$ gal (Clontech Laboratories, Palo Alto, Calif.), which contains the *Escherichia coli*  $\beta$ -galactosidase gene fused to the CMV promoter, were co-transfected as a reporter gene for monitoring the efficiency of transfection. The transfectants were analyzed for  $\beta$ -galactosidase expression by measurement of  $\beta$ -galactosidase activity [Miller (1972) *Experiments in Molecular Genetics*, pp.352-355, Cold Spring Harbor Press]. Transfectants can also be analyzed for  $\beta$ -

galactosidase expression by direct staining of the product of a reaction involving  $\beta$ -galactosidase and the X-gal substrate [Jones (1986) *EMBO* 5:3133-3142].

The efficiency of transfection of HEK cells with pCMV-KE $\alpha$ 3/pCMV-KE $\beta$ 4 was typical of standard efficiencies, whereas the efficiency of transfection of HEK cells with pCMV-KE $\alpha$ 7 was below standard levels.

## 2. Stable Transfection of HEK Cells

HEK cells were transfected using the calcium phosphate transfection procedure [*Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9 (1990)]. Ten-cm plates, each containing one-to-two million HEK cells were transfected with 1 ml of DNA/calcium phosphate precipitate containing 9.5  $\mu$ g pCMV-KE $\alpha$ 3, 9.5  $\mu$ g pCMV-KE $\beta$ 4 and 1  $\mu$ g pSV2neo (as a selectable marker). After 14 days of growth in media containing 1  $\mu$ g/ml G418, colonies had formed and were individually isolated by using cloning cylinders. The isolates were subjected to limiting dilution and screened to identify those that expressed the highest level of nicotinic AChR, as described below.

## 3. Analysis of Transfectants

### a. Fluorescent indicator-based assays

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including  $\text{Ca}^{++}$ , through the receptor channel.  $\text{Ca}^{++}$  entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic  $\text{Ca}^{++}$  levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular  $\text{Ca}^{2+}$  concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying nicotinic AChR has been described in commonly assigned pending U.S. Pat. application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

HEK cells that were transiently or stably co-transfected with DNA encoding  $\alpha$ 3 and  $\beta$ 4 subunits were analyzed for expression of functional recombinant nicotinic AChR using the automated fluorescent indicator-based assay. The assay procedure was as follows.

Untransfected HEK cells (or HEK cells transfected with pCMV-T7-2) and HEK cells that had been co-transfected with pCMV-KE $\alpha$ 3 and pCMV-KE $\beta$ 4 were plated in the wells of a 96-well microtiter dish and loaded with fluo-3 by incubation for 2 hours at 20° C. in a medium containing 20  $\mu$ M fluo-3, 0.2% Pluronic F-127 in HBS (125 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.62 mM MgSO<sub>4</sub>, 6 mM glucose, 20 mM HEPES, pH 7.4). The cells were then washed with assay buffer (i.e., HBS). The antagonist d-tubocurarine was added to some of the wells at a final concentration of 10  $\mu$ M. The microtiter dish was then placed into a fluorescence plate reader and the basal fluorescence of each well was measured and recorded before addition of 200  $\mu$ M nicotine to the wells. The fluorescence of the wells was monitored repeatedly during a period of approximately 60 seconds following addition of nicotine.

The fluorescence of the untransfected HEK cells (or HEK cells transfected with pCMV-T7-2) did not change after addition of nicotine. In contrast, the fluorescence of the co-transfected cells, in the absence of d-tubocurarine, increased dramatically after addition of nicotine to the wells. This nicotine-stimulated increase in fluorescence was not observed in co-transfected cells that had been exposed to the antagonist d-tubocurarine. These results demonstrate that the co-transfected cells express functional recombinant AChR that are activated by nicotine and blocked by d-tubocurarine.

#### b. $\alpha$ -Bungarotoxin binding assays

HEK293 cells transiently transfected with pCMV-KE $\alpha$ 7 were analyzed for [<sup>125</sup>I]- $\alpha$ -bungarotoxin (BgTx) binding. Both whole transfected cells and membranes prepared from transfected cells were examined in these assays. Rat brain membranes were included in the assays as a positive control.

Rat brain membranes were prepared according to the method of Hampson et al. (1987) *J. Neurochem* 49:1209. Membranes were prepared from the HEK cells transfected with pCMV-KE $\alpha$ 7 and HEK cells transiently transfected with plasmid pUC19 only (negative control) according to the method of Perez-Reyes et al. (1989) *Nature* 340:233. Whole transfected and negative control cells were obtained by spraying the tissue culture plates with phosphate-buffered saline containing 0.1% (w/v) BSA. The cells were then centrifuged at low speed, washed once, resuspended in assay buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 20 mM HEPES, 0.1% (w/v) BSA, 0.05% (w/v) bacitracin and 0.5 mM PMSF, pH 7.5) and counted.

Specific binding of [<sup>125</sup>I]- $\alpha$ -BgTx to rat brain membranes was determined essentially as described by Marks et al. (1982) *Molec. Pharmacol.* 22:554-564, with several modifications. The membranes were washed twice in assay buffer. The assay was carried out in 12×75 mm polypropylene test tubes in a total volume of 0.5 ml assay buffer. The membranes were incubated with 10 nM [<sup>125</sup>I]- $\alpha$ -BgTx (New England Nuclear, Boston, Mass.) for one hour at 37° C. The assay mixtures were then centrifuged at 2300×g for 10 minutes at 4× C. The supernatant was decanted and the pellets were washed twice with 2 ml aliquots of ice-cold assay buffer. The supernatants were decanted again and the radioactivity of the pellets was measured in a  $\gamma$ -counter. Non-specific binding was determined in the presence of 1  $\mu$ M unlabeled  $\alpha$ -BgTx. Specific binding was determined by subtracting nonspecific binding from total binding. Specific

binding of [ $^{125}$ I]- $\alpha$ -BgTx to membranes prepared from transfected and negative control cells was determined as described for determining specific binding to rat brain membranes except that the assay buffer did not contain BSA, bacitracin and PMSF. Specific binding of [ $^{125}$ I]- $\alpha$ -BgTx to transfected and negative control whole cells was determined basically as described for determining specific binding to rat brain membranes.

[ $^{125}$ I]- $\alpha$ -BgTx binding was evaluated as a function of membrane concentration and as a function of incubation time. [ $^{125}$ I]- $\alpha$ -BgTx binding to rat brain membranes increased in a linear fashion with increasing amounts of membrane (ranging between 25-500  $\mu$ g). The overall signal-to-noise ratio of binding (i.e., ratio of total binding to non-specific binding) was 3:1. Although some binding of [ $^{125}$ I]- $\alpha$ -BgTx to transfected cell membranes was detected, it was mostly non-specific binding and did not increase with increasing amounts of membrane. [ $^{125}$ I]- $\alpha$ -BgTx binding to the transfectants and negative control cells appeared to be similar.

To monitor [ $^{125}$ I]- $\alpha$ -BgTx binding to rat brain membranes and whole transfected and negative control cells, 300  $\mu$ g of membrane or 500,000 cells were incubated with 1 nM or 10 nM [ $^{125}$ I]- $\alpha$ -BgTx, respectively, at 37° C. for various times ranging from 0-350 min. Aliquots of assay mixture were transferred to 1.5 ml microfuge tubes at various times and centrifuged. The pellets were washed twice with assay buffer. [ $^{125}$ I]- $\alpha$ -BgTx binding to rat brain membranes increased with time and was maximal after three hours. The binding profiles of the transfected and negative control cells were the same and differed from that of rat brain membranes.

## Recombinant Expression of Human nAChR Subunits (multimeric subunit combinations) in Mammalian Cells

### II. (a) Preparation of Constructs for the Expression of Recombinant Human Neuronal Nicotinic nAChR containing multimeric Subunits.

Isolated cDNAs encoding human neuronal nAChRs were incorporated into vectors for use in expressing the subunits in mammalian host cells.

#### A. Construct for expression of a human nAChR $\alpha$ 3 subunit.

Construct pCMV-KE $\alpha$ 3 (Fig. 12) is described in U.S. Patent 5,837,489, the contents of which are incorporated by reference herein in their entirety. was digested with HindIII and NotI to release a 1.7 kb DNA fragment containing the entire  $\alpha$ 3 coding region. The expression construct pcDNA3-KE $\alpha$ 3 was prepared by ligating the 1.7 kb  $\alpha$ 3 DNA fragment from pCMV-KE $\alpha$ 3 into vector HindIII and NotI digested pcDNA3 (Invitrogen).

#### B. Construct for expression of a human nAChR $\alpha$ 5 subunit.

DNA fragments encoding portions of a human nAChR  $\alpha$ 5 subunit were ligated together to generate a full-length  $\alpha$ 5 subunit coding sequence contained in plasmid pcDNA1/Amp-KE $\alpha$ 5.5F. This construct was modified by replacing the 5'untranslated sequence of the  $\alpha$ 5 subunit DNA with a consensus ribosome binding site, RBS, (5'-GCCACC-3', see Kozak (1987)

Nucl. Acids Res. 15:8225-8148) to generate pcDNA1/Amp-KE $\alpha$ 5RBS). Construct pcDNA1/Amp-KE $\alpha$ 5RBS was digested with BamHI and EcoRI to release a 1.7 kb DNA fragment containing the consensus ribosome binding site immediately 5' to the translation initiation codon of  $\alpha$ 5 and also containing the entire  $\alpha$ 5 coding region. Construct pcDNA3-KE $\alpha$ 5RBS was prepared by digestion of pcDNA3 with BamHI and EcoRI followed by ligation of the 1.7 kb  $\alpha$ 5 DNA fragment. The pcDNA3-KE $\alpha$ 5RBS construct was then digested with Asp718I and BstXI to release a 1.7kb fragment containing the entire  $\alpha$ 5 coding sequence with the RBS immediately 5' to the  $\alpha$ 5 sequence. This fragment was ligated into expression vector pHOOK3 (Invitrogen) which had been digested with Asp718I and BstXI to generate the expression construct pHOOK3-KE $\alpha$ 5RBS (Fig. 13).

### C. Construct for expression of a human nAChR $\beta$ 2 subunit.

Construct pCMV-KE $\beta$ 2 (described in Patent 5,910,582) was modified by replacing the 5'untranslated sequence of the  $\beta$ 2 subunit DNA with a consensus ribosome binding site (5'-GCCACC-3', see Kozak (1987) Nucl. Acids Res. 15:8225-8148) to generate pCMV-KE $\beta$ 2RBS. The expression vector pCMV-KE $\beta$ 2RBS was digested with BglII and EcoRI to release a 2.2 kb DNA fragment containing the consensus ribosome binding site immediately 5' to the translation initiation codon of  $\beta$ 2 and also containing the entire  $\beta$ 2 coding region. This 2.2 kb DNA fragment was ligated into expression vector pcDNA3 that had been digested with BamHI and Eco RI. The BamHI site is compatible with BglII and this ligation generated expression construct pcDNA3-KE $\beta$ 2RBS (Fig. 14).

## II. (b) Recombinant Expression of the Human $\alpha$ 3 $\beta$ 2 $\alpha$ 5 nAChR in HEK293 cells.

Human embryonic kidney cells (HEK 293) were stably co-transfected with DNA encoding human neuronal nAChR  $\alpha$ 3,  $\beta$ 2 and  $\alpha$ 5 and analyzed for expression of nAChRs using various assays, for example, calcium sensitive fluorescent indicator-based assays and electrophysiological methods.

### 1. Stable co-transfection of HEK293 cells with human $\alpha$ 3, $\beta$ 2 and $\alpha$ 5 nAChRs.

#### a. Expression Strategy.

The  $\alpha$ 5 nAChR is non-functional when expressed with either another  $\alpha$  subunit or another  $\beta$  subunit. In order to develop a functional 3-way nAChR that includes the  $\alpha$ 5 subunit,  $\alpha$ 5 was co-expressed with both  $\alpha$ 3 and  $\beta$ 2. The antibiotic selection strategy was designed to take advantage of the lack of function of co-expression of either  $\alpha$ 3 $\alpha$ 5 or  $\alpha$ 5 $\beta$ 2. Even though these combinations would survive the antibiotic selection, they would be non-functional. Using this expression strategy, the only possible nAChR subunit combination surviving antibiotic selection and having functional responses would be  $\alpha$ 3 $\beta$ 2 $\alpha$ 5. The expression strategy for the generation of this subunit combination is described in detail below.

The  $\alpha$ 3 was cloned into pcDNA3 (Invitrogen) that encodes a neomycin resistance gene permitting tolerance to the antibiotic G418. The  $\beta$ 2 subunit was also cloned into pcDNA3. The



$\alpha 5$  subunit was cloned into the expression vector pHOOK3 (Invitrogen) which encodes the Zeocin<sup>TM</sup> (Invitrogen) resistance gene that allows tolerance to the antibiotic Zeocin<sup>TM</sup>. By this strategy, cells stably expressing the  $\alpha 5$  nAChR and  $\alpha 3$  or  $\alpha 5$  and  $\beta 2$  could survive in a selection culture medium containing both G418 and Zeocin<sup>TM</sup>. However, stable expression of  $\alpha 3$ ,  $\alpha 5$  and  $\beta 2$  would be required for function.

#### b. Recombinant Expression of Human $\alpha 3\beta 2\alpha 5$ nAChRs.

HEK293 cells were stably co-transfected with DNA encoding human neuronal nAChRs  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  using the lipofection transfection procedure (Current Protocols in Molecular Biology, Volume 1, 9.4.1 – 9.4.5 and 9.5.1 – 9.5.6, the contents of which are incorporated by reference herein). HEK293 cells were harvested and plated onto 10 cm tissue culture plates that were coated with poly-D-lysine. The HEK293 cells were plated at a concentration of 1.2 million cells per plate, 24 hours prior to transfection. Two micrograms of DNA encoding  $\alpha 3$  (mammalian expression vector pcDNA3-KE $\alpha 3$ ), 2  $\mu$ g of DNA encoding  $\beta 2$  (pcDNA3-KE $\beta 2$ RBS) and 2  $\mu$ g of DNA encoding  $\alpha 5$  (pHOOK3-KE $\alpha 5$ RBS) were diluted in 300  $\mu$ l of Dulbecco's Modified Eagle Medium (DMEM) and combined with 20  $\mu$ l of LipofectAMINE<sup>TM</sup> Reagent (Gibco-BRL) for 15 minutes. The HEK293 cells were washed twice with DMEM. This DNA/LipofectAMINE mixture was further diluted into 5.3 ml of DMEM and overlaid onto the HEK293 cells. The overlaid cells were incubated for 5 hours in an incubator at 37°C, in a humidified atmosphere containing 5% carbon dioxide. Cell plates were washed twice with 5mls of complete media (DMEM, 6% iron-supplemented calf serum, 2 mM glutamine, 100 units per ml of penicillin and 100  $\mu$ g/ml streptomycin) then overlaid with 10ml of complete medium and placed in an incubator for 48 hours.

Forty-eight hours post-transfection, cell plates were split at a 1:4 ratio, generating four culture plates. Twenty hours later, complete medium containing 100  $\mu$ g/ml of G418 plus 40  $\mu$ g/ml Zeocin<sup>TM</sup> was added to the cells for 14 days. Medium was replaced every 2 to 4 days. After this period, colonies had formed on the plates and were isolated using trypsin-soaked circles of sterile filter paper. 24 isolates were cultured, 20 survived and were expanded for functional assay using fluorescence-based measurements of internal calcium concentrations (Reference to analysis of transfectants, section 2). Two parental cell lines, 83-13 and 83-19 exhibited robust expression of the 3-way combination in functional calcium assays and both were subcloned by limiting dilution.

Thirty seven subclones from parental cell line 83-19 were screened in the fluorescence-based calcium assay. Sixteen subclones were positive in this assay and showed epibatidine-induced increases in internal calcium. Twelve subclones from parental cell line 83-13 were screened in the fluorescence-based calcium assay and five subclones were positive. Four subclones, including subclone 83-19-15 were selected based on activity in calcium assays.

83-19-15 was further subcloned by limiting dilution, and 18 subclones were screened for acetylcholine-induced increases in internal calcium. Four subclones (83-19-15-26, 83-19-15-27, 83-19-15-42 and 83-19-15-48) were selected based on a positive functional response in this assay. These subclones then entered a stability study where they were monitored for acetylcholine-induced increases in internal calcium at two-weekly intervals for approximately 15 weeks.

Subclone 83-19-15-27 was selected based on the stable functional response to low doses of acetylcholine (1  $\mu$ M) observed during the stability study. This cell line was confirmed to have acceptable responses as a random screening target in the high throughput screening assay and renamed A3B2A5 after validation in this assay (example 5, protocol A) .

## 2. Analysis of Transfectants

### a. Fluorescent indicator-based assays

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including  $\text{Ca}^{++}$ , through the receptor channel.  $\text{Ca}^{++}$  entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic  $\text{Ca}^{++}$  levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular  $\text{Ca}^{2+}$  concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying icotinic AChR has been described in commonly assigned pending U.S. Pat. application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

HEK293 cells that were stably transfected with DNA encoding the human  $\alpha 3\beta 2\alpha 5$  subunit were analyzed for expression of functional recombinant nAChRs using the automated fluorescent indicator-based assay.

Briefly, untransfected HEK293 cells and HEK293 cells that had been transfected with DNA encoding human  $\alpha 3$ ,  $\alpha 5$  and  $\beta 2$  nAChRs were plated in the wells of a poly-D-lysine coated 96-well microtiter dish at a cell density of 75,000 to 200,000 cells per well. Cells were grown in an incubator at 37°C for 2-3 hours, then transferred to an incubator maintained at 28°C. Forty-eight hours after plating, cell culture medium was decanted and cells washed with an assay buffer (HBK) containing 155 mM NaCl, 4.6 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 21.8 mM  $\text{CaCl}_2$  , 1 $\mu$ M atropine, 6 mM glucose and 20 mM HEPES-NaOH pH7.4. Washed cells were incubated with 20  $\mu$ M fluo-3-acetoxymethylester containing 0.16% pluronic F-127 at 22°C for 2 hours in the dark. Dye not taken up by cells was removed by aspiration followed by washing with 250  $\mu$ l HBK. Fluorescence measurements were performed at 0.33 sec intervals using a 96-well microtiter plate-reading fluorometer (Cambridge Technology, Inc.).

Ten basal fluorescence readings were recorded prior to addition of agonist (either 100 nM epibatidine, or 1  $\mu$ M acetylcholine). Responses after the addition of epibatidine were recorded for approximately 60 sec. Maximal fluorescence ( $F_{\max}$ ) was determined after lysing the cells with 0.25% Triton X-100, and minimal fluorescence ( $F_{\min}$ ) was determined after subsequent quenching with 10 mM  $\text{MnCl}_2$ . Calculation of  $[\text{Ca}^{2+}]_i$  was performed as described by Kao *et al.* (1989). Cellular responses were quantitated by calculating either the ratio of peak  $[\text{Ca}^{2+}]_i$  after agonist addition to the basal  $[\text{Ca}^{2+}]_i$  prior to agonist addition, or by the difference between peak  $[\text{Ca}^{2+}]_i$  and basal  $[\text{Ca}^{2+}]_i$ .

The fluorescence of the untransfected HEK cells did not change after addition of nicotine. In contrast, the fluorescence of the co-transfected cells, in the absence of d-tubocurarine, increased dramatically after addition of nicotine to the wells. This nicotine-stimulated increase in fluorescence was not observed in co-transfected cells that had been exposed to the antagonist d-tubocurarine. These results demonstrate that the co-transfected cells express the above referenced functional recombinant multimeric AChR subunit combination that were activated by nicotine and blocked by d-tubocurarine.

b. Characteristics of the stable cell line A3B2A5 that expresses the human  $\alpha 3\beta 2\alpha 5$  nAChR.

Pharmacological analysis of agonist-induced increases in internal calcium using the fura-2 calcium assay (Protocol A, *infra*, Reference to SpeedReader patent?) showed the expression of two populations of nAChRs in A3B2A5 cells: one population displayed high sensitivity to some nAChR agonists while the second showed a sensitivity to agonists indistinguishable from that observed in cell line A3B2 (which expresses human  $\alpha 3\beta 2$  nAChRs). The high affinity site in A3B2A5 cells displays a 200- to 6000-fold lower  $\text{EC}_{50}$  value for the agonists acetylcholine (ACh), nicotine and cytosine compared to  $\alpha 3\beta 2$  nAChRs. Figure 9a and 9b illustrate some of the pharmacology of the A3B2A5 cell line. The changes in agonist sensitivity result in a rank order of agonist potency for A3B2A5 that differs from that of A3B2 and thus demonstrates the presence of a novel receptor ( $\alpha 3\beta 2\alpha 5$ ) in cell line A3B2A5. In whole-cell voltage-clamped A3B2A5 cells, we found that the desensitization kinetics of currents elicited by low doses of ACh are significantly slower in A3B2A5 cells than A3B2 cells (Protocol B) (Fig. 10). The differences in biophysical properties of A3B2A5 and A3B2 also indicate the expression of a novel receptor, the  $\alpha 3\beta 2\alpha 5$  nAChR, in cell line A3B2A5 and these are illustrated in Figure 9b. The homogeneity of the cell line was verified by single-cell imaging of agonist-induced increases in intracellular free calcium concentration (Protocol C). Co-precipitation experiments demonstrated the co-assembly of the  $\alpha 5$  nAChR with  $\alpha 3$  and with  $\beta 2$  (protocol D, figure 11).

The protocols for the above referenced data is presented hereafter.

A. Fluorescence-based calcium assays using Fura-2.

A cell line, A3B2A5, stably transfected with DNAs encoding human  $\alpha 3$ ,  $\alpha 5$ , and  $\beta 2$  receptors is plated in black-walled 96-well plates, grown 2 to 3 hours at 37°C and then 2 days at 28 °C. At the start of the assay, assay the plates are washed with in HEPES buffered saline (HBS) containing 1  $\mu$ M atropine (HBSA) (wash cycle = aspirate, dispense x 3) to leave 180  $\mu$ l residual HBSA per well. Then a background measurement of a sample plate is taken by the SpeedReader

for 20 frames alternating the excitation light between 350 and 385 nm at four hertz. Twenty  $\mu$ l of 10  $\mu$ M fura-2 dye containing is then added to each well and incubated with the cells at ambient temperature for one hour to two hours. After dye loading the free dye is washed from the wells with HBSA to leave 180  $\mu$ l residual buffer per well. Two minutes after washing, a kinetic reading is taken while the test chemicals are added. The test compounds are prepared in HBSA containing 80 mM  $\text{CaCl}_2$  and 1% DMSO. The kinetic reading is composed of 140 frames, alternating between 350 and 385 as in the 20 frame background reading. However, the first 20 frames of the kinetic reading are taken before test chemical addition. The difference between these 20 frames and the background give the fluorescence due to the Ca-indicating dye fura-2. After the first 20 frames are collected 20  $\mu$ l of the test compound is dispensed from a 96-channel pipettor to the entire plate at once without halting the reading. The remainder of the 120 frames of data measure the response.

Absolute Ca concentrations are not calculated from these readings, rather the directly measured fluorescence ratio is used as a surrogate for Ca. The fluorescence ratio is calculated as dye fluorescence generated by excitation at 350 nm divided by dye fluorescence generated by excitation at 385 nm. The raw activity in a well is calculated as the maximum fluorescence ratio after compound addition divided by the average fluorescence ratio before compound addition.

## B. Electrophysiological Analysis

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques.

HEK293 cells stably transfected with DNA encoding the human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  subunits were analyzed electrophysiologically for the presence of nAChR agonist-dependent currents. HEK293 cells stably expressing human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  nAChRs were plated at a density of  $1.5 \times 10^5$  cells/35-mm dish on poly-D-lysine-coated glass coverslips (0.1 mg/ml, SIGMA) and incubated at 37°C for 2-3 hours, then for 48 hours at 28°C. Recordings were performed with an Axopatch 200A amplifier (Axon Instruments) using the whole-cell voltage-clamp configuration. Membrane potential was held at -100 mV. The standard external recording solution (mammalian Ringer's) consisted of (in mM) 160 NaCl, 5 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 11 glucose, 0.001 atropine, and 5 HEPES, pH 7.3. Ringer's solution was superfused at a rate of  $\approx 3.0$  ml/min (110  $\mu$ l recording chamber). The recording pipette solution was composed of 135 mM CsCl, 10 mM EGTA, 1 mM  $\text{MgCl}_2$  and 10 mM HEPES, pH 7.3 (with or without 4 mM Mg-ATP). Experiments were performed at room temperature. Agonist, dissolved in Ringer's solution, was applied for 200-500 ms using a fast application system, consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezo-electric drive, Winston Electronics).

The speed of solution exchange between control and nicotine-containing solutions, measured as the open-tip response, displays a time constant  $\tau=0.7$  ms, with steady state reached  $<3$  ms. Data were digitized at 6.7 kHz and filtered at 2 kHz on line. Data analysis was performed using pClamp software (Axon Instruments).

#### B. Single cell calcium imaging assays using Fura-2

Cells stably transfected with DNAs encoding human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  nAChR subunits were plated on poly-D-lysine-coated glass coverslips at a density of  $3 \times 10^5$  cells/35 mm dish and grown at 28°C. Forty-eight hours later, imaging experiments were performed at room temperature, using a Nikon TE200 inverted microscope attached to a DeltaRAM imaging System (Photon Technology International). Cells were incubated with 1  $\mu$ M fura-2-AM (Molecular Probes, Inc.) for 0.5 - 1 h and washed with mammalian Ringer's solution (see example 4, 2c for composition) to remove excess dye. Cells were transferred to a recording chamber (110  $\mu$ l, Warner Instruments), and continuously superfused with HBK containing 21.8 mM  $\text{CaCl}_2$  and 1  $\mu$ M atropine at a rate of 8 - 10 ml/min. Agonist was applied by switching between reservoirs. Cells were alternatively excited at 360 and 381 nm (0.5 Hz) to determine ratio images.

#### C. Western analysis and immunoprecipitation to demonstrate co-expression of $\alpha 3$ , $\beta 2$ and $\alpha 5$ nAChR subunit proteins.

Cells stably transfected with DNA encoding human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  nAChR subunits were harvested from 10-cm plates and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4). Washed cells were resuspended in 50 mM Tris pH 7.4, 1 mM EDTA containing a cocktail of protease inhibitors (Complete<sup>TM</sup>, Boehringer Mannheim, Indianapolis, IN) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000 x g for 5 min to remove cellular debris, and the supernatant fraction was centrifuged at 100,000 x g for 120 min to pellet the membranes. The membranes were resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1% SDS) containing protease inhibitor cocktail.

For immunoprecipitation experiments, 200  $\mu$ g of membranes were immunoprecipitated with 20  $\mu$ g of a sheep anti-rat  $\alpha 3$  polyclonal antibody (Bethyl Laboratories, or 2  $\mu$ g a rabbit anti-human  $\beta 2$  polyclonal antibody (MRL San Diego) overnight at 4°C. The antibody-antigen complexes were affinity-purified using Protein G sepharose, incubated overnight at 4°C then solubilized in SDS sample buffer. For immunoblot analysis, membranes were solubilized in Tris-Glycine SDS Sample Buffer (Novex) containing 5% 2-mercaptoethanol and heated at 65°C for 10 min. Solubilized proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hy-Bond ECL, Amersham, Arlington Heights, IL). Blots were rinsed once in PBS, 0.1% Tween-20 (wash buffer), then blocked for 3 h in 5% Carnation non-fat dry milk dissolved in wash buffer (blocking buffer).

The human  $\alpha 5$  protein was detected with a sheep anti-rat  $\alpha 5$  antibody (Bethyl Laboratories). The  $\alpha 5$  antibody was diluted to 15  $\mu$ g/ml in blocking buffer and incubated with the nitrocellulose

membrane for 3 h at room temperature. The membranes were washed three times in wash buffer. The secondary antibody was peroxidase-conjugated donkey anti-sheep IgG (Cappel Antibodies) diluted 1:1000 in blocking buffer and incubated with membranes for 45 min at room temperature, followed by five changes of wash buffer. The antibody signal was visualized using the ECL developing system (Amersham) according to the manufacturer's directions.

The above strategy may be employed in expressing any one of the following multimeric subunit combinations of the alpha and beta subunit of nAChR, especially when the nucleic acid molecule encoding each individual nAChR subunit is disclosed herein. In view of the above data, it is not seen why the proposed combinations appearing below would not act in a manner similar to the multimeric subunit combination discussed immediately above.

$\alpha_2\beta_4\alpha_6$

$\alpha_3\beta_4\alpha_6$

$\alpha_4\beta_4\alpha_5$

$\alpha_4\beta_4\alpha_6$

$\alpha_4\beta_2\alpha_5$

$\alpha_4\beta_2\beta_3$

$\alpha_3\beta_2\alpha_6\beta_3$

$\alpha_2\beta_4\alpha_5$

$\alpha_2\beta_2\alpha_5$

$\alpha_X\beta_2\beta_4$ , where X refers to one or more of the  $\alpha$  subunits disclosed herein.

$\alpha_X\beta_2\beta_3\beta_4$ , where X refers to one or more of the  $\alpha$  subunits disclosed herein

$\alpha_X\beta_2\beta_3$ , where X refers to one or more of the  $\alpha$  subunits disclosed herein

$\alpha_2\beta_2\alpha_6$

$\alpha_3\beta_2\alpha_6$

$\alpha_4\beta_2\alpha_6$

Five-way combinations of subunits, represented by the general formula  $\alpha_n\beta_m$ , wherein n and m are each 0-5 (where the  $\alpha$  subunit is one or more of  $\alpha_1$  thru  $\alpha_7$  and  $\beta$  is any one or more of  $\beta_2$ ,  $\beta_3$  or  $\beta_4$  are also contemplated by the present invention. Likewise, four-way combinations are also a feature of the invention.

### III. Recombinant Expression of the Human $\alpha_7$ nAChR in a non-human cell line

#### A. Construct for Expression of recombinant Human nAChR $\alpha_7$ in a Non-human Host Cell Line

The isolated cDNA encoding human neuronal  $\alpha_7$  AChR was incorporated into the expression vector pcDNA3 (Invitrogen) for use in expressing the  $\alpha_7$  subunit in the GH<sub>4</sub>C<sub>1</sub> host cell line. The expression vector, pcDNA3-KE $\alpha_7$ RBS was constructed as described below.

Construct pCMV-KE $\alpha_7$  was digested with BamHI and XhoI to release a 1.8kb DNA fragment containing a consensus ribosome binding site (RBS) immediately 5' to the translation initiation codon of  $\alpha_7$  and also containing the entire  $\alpha_7$  coding region. pGEM/KE $\alpha_7$ RBS was prepared by

ligating this 1.8kb DNA fragment into BamHI, XhoI digested pGEM-7Zf(+), (Promega). pGEM/KE $\alpha$ 7RBS was digested with BamHI and XhoI to release the 1.8kb DNA fragment containing the RBS and  $\alpha$ 7 coding region. pcDNA3-KE $\alpha$ 7RBS was prepared by ligating the 1.8kb fragment from pGEM/KE $\alpha$ 7RBS into BamHI and XhoI digested pcDNA3.

#### B. Recombinant Expression of the Human $\alpha$ 7 nAChR in GH $_4$ C $_1$ cells.

GH $_4$ C $_1$  cells, derived from rat pituitary tumor tissue, were stably transfected with DNA encoding human neuronal nAChR  $\alpha$ 7 and analyzed for expression of nAChRs using various assays, for example calcium sensitive fluorescent indicator-based assays, [ $^{125}$ I] bungarotoxin binding and electrophysiological methods.

##### 1. Stable Transfection of GH $_4$ C $_1$ cells with the human $\alpha$ 7 nAChR.

GH $_4$ C $_1$  cells were stably transfected with DNA encoding human neuronal nAChR  $\alpha$ 7 using the lipofection transfection procedure (Current Protocols in Molecular Biology, Volume 1, 9.4.1 – 9.4.5 and 9.5.1 – 9.5.6, incorporated herein by reference).

GH $_4$ C $_1$  cells were harvested using Cell Dissociation Buffer (Sigma) and plated onto 10 cm tissue culture plates coated with poly-d-lysine at a concentration of 1.2 million cells per plate, 24 hours prior to transfection. Six micrograms of the  $\alpha$ 7 expression vector, pcDNA3-KE $\alpha$ 7RBS were diluted in 300  $\mu$ l of Dulbecco's Modified Eagle Medium (DMEM) and combined with 20  $\mu$ l of LipofectAMINE<sup>TM</sup> Reagent (Gibco-BRL) for 15 minutes. The GH $_4$ C $_1$  cells were washed twice with DMEM. This DNA/LipofectAMINE mixture was further diluted into 5.3 ml of DMEM and overlaid onto the GH $_4$ C $_1$  cells. The overlaid cells were incubated for 5 hours in an incubator at 37°C, in a humidified atmosphere containing 6% carbon dioxide. Cell plates were washed twice with 5mls of Ham's F-10 nutrient mixture (GibcoBRL) containing 10% fetal bovine serum, 100 units per ml of penicillin and 100  $\mu$ g/ml streptomycin then overlaid with 10ml of complete medium and placed in an incubator for 48 hours.

Forty-eight hours post-transfection, cell plates were split at a 1:4 ratio, generating four culture plates. Twenty hours later, complete medium containing 500  $\mu$ g/ml of G418 was added to the cells for 14 days. Medium was replaced every 2 to 4 days. After this period, colonies had formed on the plates and were isolated using trypsin-soaked circles of sterile filter paper. 24 isolates were cultured, 18 survived and were expanded for functional assay using fluorescence-based measurements of internal calcium concentrations as described in Example 4 above.

Clones were also screened in a radioligand binding assay using [ $^{125}$ I]-bungarotoxin. See example 4. Electrophysiological recordings (similar to the procedure outlined in Example 4) also demonstrated currents with biophysical properties characteristic of the  $\alpha$ 7 receptor. Parental cell line G1-9 exhibited robust expression in both functional calcium and electrophysiological assays and in binding assays. The G1-9 parental cell line was subcloned by limiting dilution.

Twenty eight subclones from G1-9 were screened in the fluorescence-based calcium assay. Ten subclones were positive in this assay and showed epibatidine-induced increases in internal

calcium. An additional binding assay, similar to that outlined above, identified thirteen positive subclones.

Five subclones, including subclone G1-19-15 were selected based on activity in both calcium and binding assays.

G1-9-15 was further subcloned by limiting dilution, subclones were screened for epibatidine-induced increases in internal calcium. Four subclones, G1-9-15-8, G1-9-15-18, G1-9-15-28 and G1-9-15-35 were selected based on a positive functional response in this assay. These subclones then entered a stability study where they were monitored for functional response in the calcium assay at two-weekly intervals for approximately 15 weeks.

Subclone G1-9-15-8 was selected based on the stable functional response observed during the stability study. This cell line was confirmed to have acceptable responses as a random screening target in the high throughput screening assay and renamed A7 after validation in this assay.

## 2. Analysis of Transfectants

### a. Fluorescence-based measurements of internal calcium concentrations.

GH<sub>4</sub>C<sub>1</sub> cells that were stably transfected with DNA encoding the human  $\alpha 7$  subunit were analyzed for expression of functional recombinant nAChRs using the automated fluorescent indicator-based assay.

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including Ca<sup>++</sup>, through the receptor channel. Ca<sup>++</sup> entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic Ca<sup>++</sup> levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular Ca<sup>2+</sup> concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying nicotinic AChR has been described in commonly assigned pending U.S. Pat. application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

Untransfected GH<sub>4</sub>C<sub>1</sub> cells and GH<sub>4</sub>C<sub>1</sub> cells that had been transfected with pcDNA3-KE $\alpha 7$ RBS were plated in the wells of a poly-D-lysine coated 96-well microtiter dish at a cell density of



75,000 to 200,000 cells per well. Twenty four hours after plating, cell culture medium was decanted and cells washed with an assay buffer (HBK) containing 155 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 1 μM atropine, 6 mM glucose and 20 mM Hepes-NaOH pH7.4. Washed cells were incubated with 20 μM fluo-3-acetoxymethylester containing 0.16% pluronic F-127 at 22°C for 2 hours in the dark. Dye not taken up by cells was removed by aspiration followed by washing with 250 μl HBK. Fluorescence measurements were performed at 0.33 sec intervals using a 96-well microtiter plate-reading fluorometer (Cambridge Technology, Inc.). Cells were incubated for 10 minutes with 3 μM FPL 64176 and ten basal fluorescence readings were recorded prior to addition of 1 μM epibatidine. Responses after the addition of epibatidine were recorded for approximately 60 sec. Alpha-bungarotoxin was tested after a preincubation period of 5 - 10 min. Maximal fluorescence ( $F_{max}$ ) was determined after lysing the cells with 0.25% Triton X-100, and minimal fluorescence ( $F_{min}$ ) was determined after subsequent quenching with 10 mM MnCl<sub>2</sub>. Calculation of  $[Ca^{2+}]_i$  was performed as described by Kao *et al.* (1989). Cellular responses were quantitated by calculating either the ratio of peak  $[Ca^{2+}]_i$  after agonist addition to the basal  $[Ca^{2+}]_i$  prior to agonist addition, or by the difference between peak  $[Ca^{2+}]_i$  and basal  $[Ca^{2+}]_i$ .

#### b. α-Bungarotoxin Binding Assays

Untransfected GH<sub>4</sub>C<sub>1</sub> cells and GH<sub>4</sub>C<sub>1</sub> cells that were stably transfected with DNA encoding the human α7 subunit were analyzed for [<sup>125</sup>I]-α-bungarotoxin binding. The assay procedure was as follows.

Cells were incubated with 1nM [<sup>125</sup>I]-α BTX in culture media for 2 hours at room temperature. Non-specific binding was determined in the presence of 1 μM unlabeled toxin. The assays were terminated by aspiration of the culture media and rapid filtration through Whatman GF/C filters using a Brandel Cell Harvester. Filters were washed with approximately 4x1ml washes of ice cold binding assay buffer (50 mM tris, 140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4). Filter disks were transferred to scintillation vials containing 5 ml Ecolume scintillation cocktail and radioactivity counted using a Beckman 6500 scintillation spectrometer.

#### c. Electrophysiological Analysis of GH<sub>4</sub>C<sub>1</sub> cells Transfected with Human Neuronal Nicotinic AChR Subunit-encoding DNA (human α7 subunit)

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques.

GH<sub>4</sub>C<sub>1</sub> cells stably transfected with DNA encoding the human α<sub>7</sub> subunit were analyzed electrophysiologically for the presence of nAChR agonist-dependent currents. GH<sub>4</sub>C<sub>1</sub> cells stably expressing human α<sub>7</sub> nAChRs were plated at a density of 1.5 x 10<sup>5</sup> cells/35-mm dish on

collagen-coated glass coverslips (rat collagen I, Becton Dickinson) treated with an additional coating of poly-D-lysine (0.1 mg/ml, SIGMA). Recordings were performed with an Axopatch 200A amplifier (Axon Instruments) using the whole-cell voltage-clamp configuration. Membrane potential was held at -100 mV. The standard external recording solution (mammalian Ringer's) consisted of (in mM) 160 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 11 glucose, 0.001 atropine, and 5 HEPES, pH 7.3. Ringer's solution was superfused at a rate of  $\approx 3.0$  ml/min (110  $\mu$ l recording chamber). The recording pipette solution was composed of 135 mM CsCl, 10 mM EGTA, 1 mM MgCl<sub>2</sub> and 10 mM HEPES, pH 7.3 (with or without 4 mM Mg-ATP). Experiments were performed at room temperature. Nicotine (100-300  $\mu$ M), dissolved in Ringer's solution, was applied for 200-500 ms using a fast application system, consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezo-electric drive, Winston Electronics). The speed of solution exchange between control and nicotine-containing solutions, measured as the open-tip response, displays a time constant  $\tau=0.7$  ms, with steady state reached  $<3$  ms. Data were digitized at 6.7 kHz and filtered at 2 kHz on line. Data analysis was performed using pClamp software (Axon Instruments).

## EXAMPLE 5

### Characterization of Cell Lines Expressing nAChRs

Recombinant cell lines generated by transfection with DNA encoding human neuronal nicotinic AChRs, such as those described in Example 3 can be further characterized using one or more of the following methods.

#### A. Northern or slot blot analysis for expression of $\alpha$ - and/or $\beta$ -subunit encoding messages

Total RNA is isolated from  $\sim 1 \times 10^7$  cells and 10-15  $\mu$ g of RNA from each cell type is used for northern or slot blot hybridization analysis. The inserts from human neuronal nAChR-encoding plasmids can be nick-translated and used as probe. In addition, the  $\beta$ -actin gene sequence (Cleveland et al. (1980) Cell 20:95-105) can be nick-translated and used as a control probe on duplicate filters to confirm the presence or absence of RNA on each blot and to provide a rough standard for use in quantitating differences in  $\alpha$ - or  $\beta$ -specific mRNA levels between cell lines. Typical northern and slot blot hybridization and wash conditions are as follows:

hybridization in 5 $\times$  SSPE, 5 $\times$  Denhardt's solution, 50% formamide, at 42° C. followed by washing in 0.2 $\times$  SSPE, 0.1% SDS, at 65° C.

#### B. Nicotine-binding assay

Cell lines generated by transfection with human neuronal nicotinic AChR  $\alpha$ - or  $\alpha$ - and  $\beta$ -subunit-encoding DNA can be analyzed for their ability to bind nicotine, for example, as compared to control cell lines: neuronally-derived cell lines PC12 (Boulter et al., (1986), supra; ATCC #CRL1721) and IMR32 (Clementi, et al. (1986); Int. J. Neurochem. 47:291-297; ATCC #CCL127), and muscle-derived cell line BC3H1 (Patrick, et al, (1977); J. Biol. Chem. 252:2143-2153. Negative control cells (i.e., host cells from which the transfectants were prepared) are also included in the assay. The assay is conducted as follows:

Just prior to being assayed, transfected cells are removed from plates by scraping. Positive control cells used are PC12, BC3H1, and IMR32 (which had been starved for fresh media for seven days). Control cell lines are removed by rinsing in 37° C. assay buffer (50 mM Tris/HCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 120 mM NaCl, 3 mM EDTA, 2 mg/ml BSA and 0.1% aprotinin at pH7.4). The cells are washed and resuspended to a concentration of 1×10<sup>6</sup>/250 μl. To each plastic assay tube is added 250 μl of the cell solution, 15 nM <sup>3</sup>H-nicotine, with or without 1 mM unlabeled nicotine, and assay buffer to make a final volume of 500 μl. The assays for the transfected cell lines are incubated for 30 min at room temperature; the assays of the positive control cells are incubated for 2 min at 1° C. After the appropriate incubation time, 450 μl aliquots of assay volume are filtered through Whatman GF/C glass fiber filters which has been pretreated by incubation in 0.05% polyethyleneimine for 24 hours at 4° C. The filters are then washed twice, with 4 ml each wash, with ice cold assay buffer. After washing, the filters are dried, added to vials containing 5 ml scintillation fluid and radioactivity is measured.

### C. <sup>86</sup>Rb ion-flux assay

The ability of nicotine or nicotine agonists and antagonists to mediate the influx of <sup>86</sup>Rb into transfected and control cells has been found to provide an indication of the presence of functional AChRs on the cell surface. The <sup>86</sup>Rb ion-flux assay is conducted as follows:

1. The night before the experiment, cells are plated at 2×10<sup>6</sup> per well (i.e., 2 ml per well) in a 6-well polylysine-coated plate.
2. The culture medium is decanted and the plate washed with 2 ml of assay buffer (50 mM HEPES, 260 mM sucrose, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 5.5 mM glucose) at room temperature.
3. The assay buffer is decanted and 1 ml of assay buffer, containing 3 μCi/ml <sup>86</sup>Rb, with 5 mM ouabain and agonist or antagonist in a concentration to effect a maximum response, is added.
4. The plate is incubated on ice at 1° C. for 4 min.
5. The buffer is decanted into a waste container and each well was washed with 3 ml of assay buffer, followed by two washes of 2 ml each.
6. The cells are lysed with 2×0.5 ml of 0.2% SDS per well and transferred to a scintillation vial containing 5 ml of scintillation fluid.
7. The radioactivity contained in each vial is measured and the data calculated.

Positive control cells provided the following data in this assay:

PC12	IMR32
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	EC <sub>50</sub>	Maximum response	EC <sub>50</sub>	Maximum response
<u>Agonist</u>				
nicotine	52 $\mu$ M	2.1 X <sup>a</sup>	18 $\mu$ M	7.7 X <sup>a</sup>
CCh*	35 $\mu$ M	3.3 X <sup>b</sup>	230 $\mu$ M	7.6 X <sup>c</sup>
cytisine	57 $\mu$ M	3.6 X <sup>d</sup>	14 $\mu$ M	10 X <sup>e</sup>
<u>Antagonist</u>				
d-tubocurarine	0.81 $\mu$ M		2.5 $\mu$ M	
mecamylamine	0.42 $\mu$ M		0.11 $\mu$ M	
hexamethonium	nd <sup>f</sup>		22 $\mu$ M	
atropine	12.5 $\mu$ M		43 $\mu$ M	

\*CCh = carbamylcholine

<sup>a</sup>200  $\mu$ M nicotine

<sup>b</sup>300  $\mu$ M CCh

<sup>c</sup>3 mM CCh

<sup>d</sup>1 mM cytisine

<sup>e</sup>100  $\mu$ M cytisine

<sup>f</sup>nd = not determined

#### D. Electrophysiological Analysis of Mammalian Cells Transfected with Human Neuronal Nicotinic AChR Subunit-encoding DNA

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques. In preferred embodiments, transfected mammalian cells or injected oocytes are analyzed electrophysiologically for the presence of AChR agonist-dependent currents.

#### Example 6

##### Characterization of GH<sub>4</sub>C<sub>1</sub> Cells Stably Expressing the Human $\alpha$ 7 nAChR

The cell line A7 that stably expressed the human  $\alpha$ 7 nAChR was characterized in multiple assays that are described below.

Dose response curves to reference nicotinic agonists nicotine and acetylcholine were obtained for cell line A7 using the fura-2 based calcium assay. See protocol A *infra*.

Referring to Figure 3, the EC<sub>50</sub> for nicotine was 2  $\mu$ M and for acetylcholine was 7  $\mu$ M. This is in agreement with that reported for the  $\alpha$ 7 nAChR ( Peng et al (1993) Mol Pharmacol. 45:546-554).

Data on electrophysiological characterization using whole-cell voltage-clamped A7 cells is depicted in Figure 4, which show rapidly desensitizing currents that are consistent with those reported for  $\alpha$ 7 nAChRs. The protocols for these experiments were the same as those described in Examples 3 and 4 above. In these studies 90% to 100% of voltage-clamped A7 cells responded to the application of 300  $\mu$ M nicotine.

Single cell calcium imaging of the A7 cell line (Figure 6) (protocol B, *infra*) supports the conclusion that individual cells in this cell line (A7) respond to 10  $\mu$ M epibatidine in a homogenous manner.

In radioligand binding studies (protocol C, *infra*) methyllycaconitine (MLA) displaced [<sup>3</sup>H]-MLA binding from the  $\alpha$ 7 nAChRs in cell line A7 with an IC<sub>50</sub> of 4 nM, similar to the IC<sub>50</sub> value obtained with  $\alpha$ -bungarotoxin (3 nM). These IC<sub>50</sub> values are similar to published affinities (for example, Davies *et al.* 1999, Neuropharmacology 38:679).  $\alpha$ -bungarotoxin displaced approximately 65% of the [<sup>3</sup>H]-MLA binding in A7. Cells are permeable to MLA but not to  $\alpha$ -bungarotoxin under these assay conditions. This therefore demonstrates that 65% of the  $\alpha$ 7 nAChRs in cell line A7 are expressed on the plasma membrane (i.e. at the cell surface). This data is illustrated in Figure 5.

A molecular characterization was undertaken to demonstrate the expression of  $\alpha$ 7 nAChR protein and  $\alpha$ 7 mRNA in the stable cell line A7. Western analysis using an  $\alpha$ 7-specific antibody demonstrated that cell line A7 expressed protein of approximately 54 kDa. Protein prepared from the untransfected GH<sub>4</sub>C<sub>1</sub> cell line does not show any hybridization with this antibody. Refer to Figure 7.

Northern analysis of total RNA prepared from A7 cells showed that these cells express an RNA species that hybridizes with a subunit specific DNA probe. The hybridizing band has a molecular weight of approximately 2.4 kb. No hybridizing species was detected in untransfected GH<sub>4</sub>C<sub>1</sub> cells. Refer to Figure 8.

The characterizations of stable cell line A7 described above were generated using the following protocols .

#### A. Fluorescence based calcium assays using Fura-2

A cell line A7 stably transfected with the human  $\alpha$ 7 nAChR receptor is plated in black-walled 96-well plates and grown at 37°C. Twenty-four hours later, the plates are washed with in HEPES buffered saline (HBS) containing 1  $\mu$ M atropine (HBSA) (wash cycle = aspirate, dispense x 3) to

leave 180  $\mu$ l residual HBSA per well. At the start of the assay, a background measurement of a sample plate was taken by the SpeedReader for 20 frames alternating the excitation light between 350 and 385 nm at four hertz. See U.S. Patent number 5,670,113 and 6,057,114, each of which is incorporated by reference herein in their entirety. Twenty  $\mu$ l of 10  $\mu$ M fura-2 dye containing 3  $\mu$ M FPL-64176 is then added to each well and incubated with the cells at ambient temperature for one to two hours. After dye loading the free dye is washed from the wells with HBSA containing 0.5  $\mu$ M FPL-64176 to leave 180  $\mu$ l residual buffer per well. Two minutes after washing, a kinetic reading is taken while the test chemicals are added. The test compounds are prepared in HBSA containing 80 mM CaCl<sub>2</sub> and 1% DMSO. The kinetic reading is composed of 140 frames, alternating between 350 and 385 as in the 20 frame background reading. However, the first 20 frames of the kinetic reading are taken before test chemical addition. The difference between these 20 frames and the background give the fluorescence due to the calcium-indicating dye fura-2. After the first 20 frames are collected 20  $\mu$ l of the test compound is dispensed from a 96-channel pipettor to the entire plate at once without halting the reading. The remainder of the 120 frames of data measure the response.

Absolute calcium concentrations are not calculated from these readings, rather the directly measured fluorescence ratio is used as a surrogate for calcium. The fluorescence ratio is calculated as dye fluorescence generated by excitation at 350 nm divided by dye fluorescence generated by excitation at 385 nm. The raw activity in a well is calculated as the maximum fluorescence ratio after compound addition divided by the average fluorescence ratio before compound addition.

#### B. Single cell calcium imaging assays using Fura-2

Cells stably transfected with the human  $\alpha$ 7 nAChR were plated on poly-D-lysine-coated glass coverslips at a density of  $3 \times 10^5$  cells/35 mm dish. Twenty four hours later, imaging experiments were performed at room temperature, using a Nikon TE200 inverted microscope attached to a DeltaRAM imaging System (Photon Technology International). Cells were incubated with 1 $\mu$ M fura-2-AM (Molecular Probes, Inc.) for 0.5 - 1 h and washed with mammalian Ringer's solution (see example above re: the ephys composition of this buffer eg Ringers (in mM) 160 NaCl, 5 KCl, 1 MgCl etc.) to remove excess dye. Cells were transferred to a recording chamber (110  $\mu$ l, Warner Instruments), and continuously superfused with HBK containing 1 $\mu$ M atropine at a rate of 8 - 10 ml/min. 10 $\mu$ M epibatidine was applied by switching between reservoirs. Cells were alternatively excited at 360 and 381 nm (0.5 Hz) to determine ratio images.

#### C. Radioligand Binding Studies

GH4C1 cells stably expressing  $\alpha$ 7 were plated in 96-well microtiter plates at a density of 200,000 cells per well. Twenty-four hours later, cells were washed in assay buffer (50 mM Tris, 140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4), and incubated with 1 nM [<sup>3</sup>H]-methyllycaconitine in the presence of increasing concentrations of either methyllycaconitine (MLA) or  $\alpha$ -bungarotoxin. After 120 min, the assay was terminated by aspiration of the buffer and rapid filtration through Whatman GF/C filters using a Brandel Cell Harvester. Filters were washed with approximately 4 x 1ml washes of ice cold assay buffer, and filter disks transferred to scintillation vials containing 5 ml Ecolume scintillation cocktail. Radioactivity was counted

using a Beckman 6500 scintillation spectrometer. Specific binding was calculated by subtracting the non-specific binding, defined by 10  $\mu$ M MLA.

#### D. Western analysis for expression of $\alpha 7$ protein

Cells stably transfected with the human  $\alpha 7$  nAChR were harvested from 10-cm plates and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4). Washed cells were resuspended in 50 mM Tris pH 7.4, 1 mM EDTA containing a cocktail of protease inhibitors (Complete<sup>TM</sup>, Boehringer Mannheim, Indianapolis, IN) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000 x g for 5 min to remove cellular debris, and the supernatant fraction was centrifuged at 100,000 x g for 120 min to pellet the membranes. The membranes were resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1% SDS) containing protease inhibitor cocktail.

For immunoblot analysis, membranes were solubilized in Tris-Glycine SDS Sample Buffer (Novex, San Diego, CA) containing 5% 2-mercaptoethanol and heated at 65°C for 10 min. Solubilized proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hy-Bond ECL, Amersham, Arlington Heights, IL). Blots were rinsed once in PBS, 0.1% Tween-20 (wash buffer), then blocked for 3 h in 5% Carnation non-fat dry milk dissolved in wash buffer (blocking buffer).

The human  $\alpha 7$  protein was detected with an antibody raised in goat against a human  $\alpha 7$ -specific peptide (Santa Cruz Biotechnology). The primary antibody was diluted to 0.5  $\mu$ g/ml in blocking buffer and incubated with the nitrocellulose membrane for 3 h at room temperature. The membranes were washed three times in wash buffer. The secondary antibody was peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) diluted 1:2500 in blocking buffer and incubated with membranes for 45 min at room temperature, followed by five changes of wash buffer. The antibody signal was visualized using the ECL developing system (Amersham) according to the manufacturer's directions.

#### E. Northern analysis for expression of $\alpha 7$ encoding message.

Total RNA was isolated from approximately  $1 \times 10^7$  cells for northern hybridization analysis. Total RNA was size-fractionated on an agarose-formaldehyde gel and blotted to nylon by downward alkaline transfer. Blots were hybridized with digoxigenin-labeled DNA probes specific for human  $\alpha 7$  subunits (nucleic acid numbers 1066-1533). Blots were hybridized overnight with 20 ng/ml probe and washed at high stringency in a wash buffer containing 0.1X SSPE (3mM NaCl, 0.2mM  $\text{NaH}_2\text{PO}_4$ , 0.02mM EDTA) and 0.1% SDS at 65°C. Chemiluminescent detection was performed using the Genius 7 kit (Boehringer Mannheim) according to the manufacturer's instructions. Refer to Figure 8.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

## Summary of Sequences

Sequence ID No. 1 is a nucleotide sequence encoding an  $\alpha_2$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 2 is the amino acid sequence of the  $\alpha_2$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 1.

Sequence ID No. 3 is a nucleotide sequence encoding an  $\alpha_3$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 4 is the amino acid sequence of the  $\alpha_3$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 3.

Sequence ID No. 5 is a nucleotide sequence encoding an  $\alpha_4$  subunit of a human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 6 is the amino acid sequence of the  $\alpha_4$  subunit of a human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 5.

Sequence ID No. 7 is a nucleotide sequence encoding an  $\alpha_5$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 8 is the amino acid sequence of the  $\alpha_5$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 7.

Sequence ID No. 9 is a nucleotide sequence encoding an  $\alpha_6$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 10 is the amino acid sequence of the  $\alpha_6$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 9.

Sequence ID No. 11 is the nucleotide sequence encoding an  $\alpha_7$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 7.

Sequence ID No. 12 is the amino acid sequence of the  $\alpha_7$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 11.

Sequence ID No. 13 is a nucleotide sequence encoding a  $\beta_2$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 14 is the amino acid sequence of the  $\beta_2$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 9.



Sequence ID No. 15 is a nucleotide sequence encoding a  $\beta_3$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 16 is the amino acid sequence of the  $\beta_3$  subunit of human neuronal nicotinic acetylcholine receptor, set forth in Sequence ID No. 15.

Sequence ID No. 17 is a nucleotide sequence encoding a  $\beta_4$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 18 is the amino acid sequence of the  $\beta_4$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 17.

## Claims

We claim:

1. A suitable host cell transfected with an isolated nucleic acid molecule comprising a sequence of nucleotides or ribonucleotides that encodes an  $\alpha_7$  subunit of a human neuronal nicotinic acetylcholine receptor.
2. A recombinant non-human *cell line* which has been engineered to express a heterologous protein, said cell line comprising a host cell transformed or transfected with a heterologous nucleic acid molecule comprising a sequence of nucleotides or ribonucleotides that inducibly express an  $\alpha_7$  subunit of a human neuronal nicotinic acetylcholine receptor.
3. The cell line according to claim 1, wherein the cell line is derived from rat pituitary tumour tissue.
4. The cell line according to claim 1 wherein the heterologous protein is a functional human neuronal nicotinic acetylcholine receptor.
5. The cell line according to claim 1, further comprising a marker gene, wherein expression of the marker gene indicates expression of the heterologous protein.
6. The cell line according to claim 1, wherein the heterologous nucleic acid molecule is contained within an expression vector.
7. An isolated cell transformed or transfected with a sequence of nucleotides or ribonucleotides under conditions favoring cell surface expression of a functional neuronal nicotinic acetylcholine receptor that contains at least one  $\alpha$  and at least one  $\beta$  subunit.
8. The isolated cell according to claim 7 wherein the isolated cell expresses a functional neuronal nicotinic acetylcholine receptor that comprises a multimeric subunit combination, wherein said multimeric subunit combination is defined by the general formula  $\alpha_X\beta_Y\beta_Z$ , where X is at least one member selected from the group consisting of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_6$  and  $\alpha_7$ ; Y is at least one member selected from the group consisting of  $\beta_2$ ,  $\beta_3$  and  $\beta_4$  and Z is selected from the group consisting of  $\beta_2$ ,  $\beta_3$  and  $\beta_4$ .
9. The isolated cells according to claim 8 wherein the functional neuronal nicotinic acetylcholine receptor contains at least one of the following subunit combinations selected from the group consisting of  $\alpha_2\beta_4\alpha_6$ ;  $\alpha_3\beta_4\alpha_6$ ;  $\alpha_4\beta_4\alpha_5$ ;  $\alpha_4\beta_4\alpha_6$ ;  $\alpha_4\beta_2\alpha_5$ ;  $\alpha_4\beta_2\beta_3$ ;  $\alpha_3\beta_2\alpha_6\beta_3$ ;  $\alpha_2\beta_4\alpha_5$ ;  $\alpha_2\beta_2\alpha_5$ ;  $\alpha_3\beta_2\alpha_5$ ;  $\alpha_3\beta_4\alpha_5$ .
10. The isolated cells according to claim 8 wherein the functional neuronal nicotinic acetylcholine receptor contains at least one of the following subunit combinations selected from the group consisting of  $\alpha_2\beta_2\alpha_6$ ;  $\alpha_3\beta_2\alpha_6$ ;  $\alpha_4\beta_2\alpha_6$ .

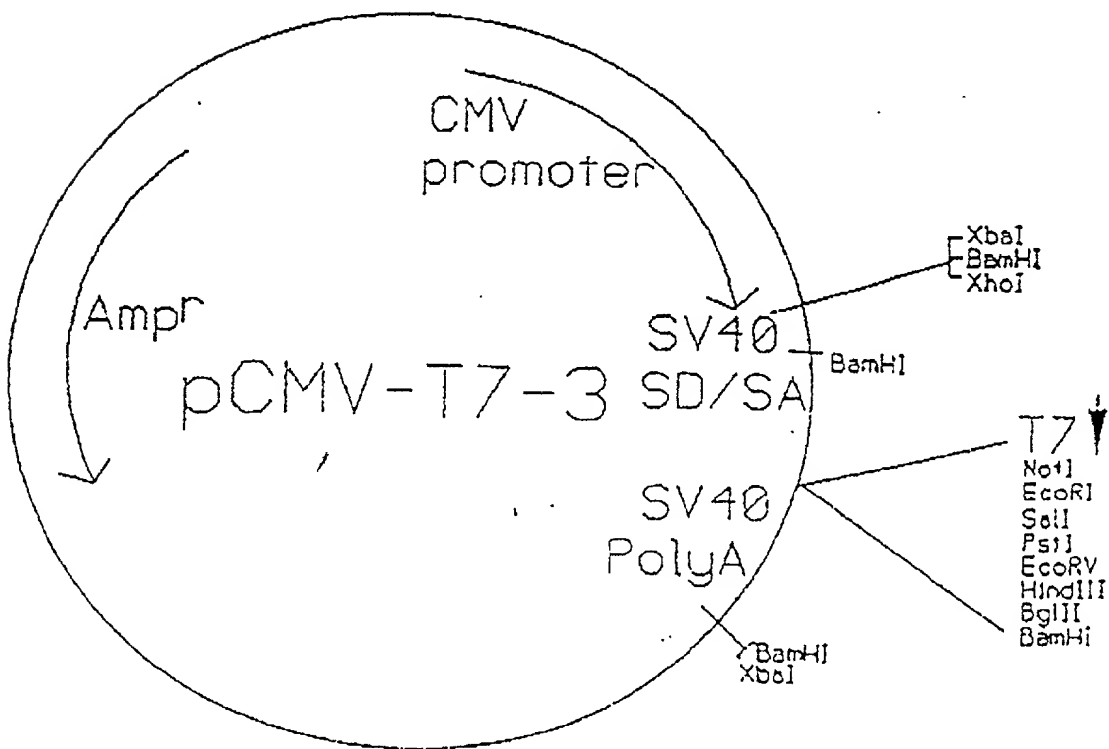
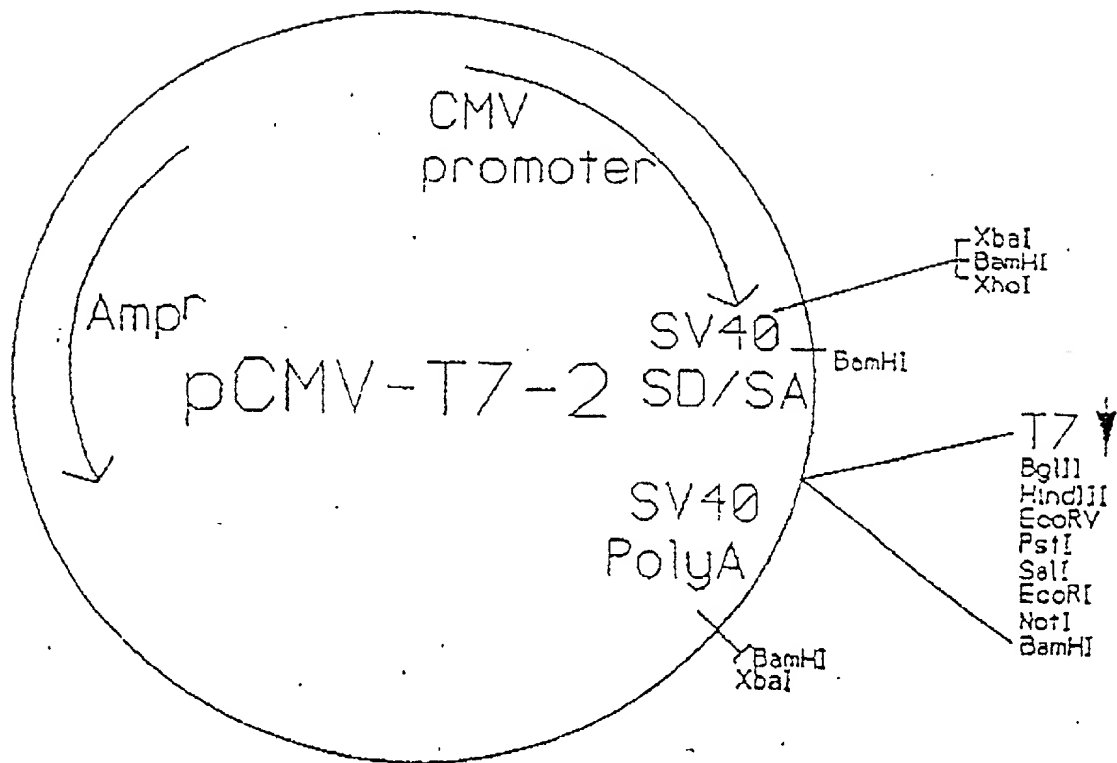
11. The isolated cell according to 8 wherein said cell expresses a functional neuronal nicotinic acetylcholine receptor that comprises a multimeric subunit combination, wherein said multimeric subunit combination is defined by the general formula  $\alpha_X\beta_2\beta_4$ , where X is one or more of  $\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5, \alpha_6$  or  $\alpha_7$ .

12. The isolated cells according to 8 claim wherein the functional neuronal nicotinic acetylcholine receptor contains a multimeric subunit combination defined by the formula  $\alpha_X\beta_2\beta_3$ , where X is one or more of  $\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5, \alpha_6$  or  $\alpha_7$ .

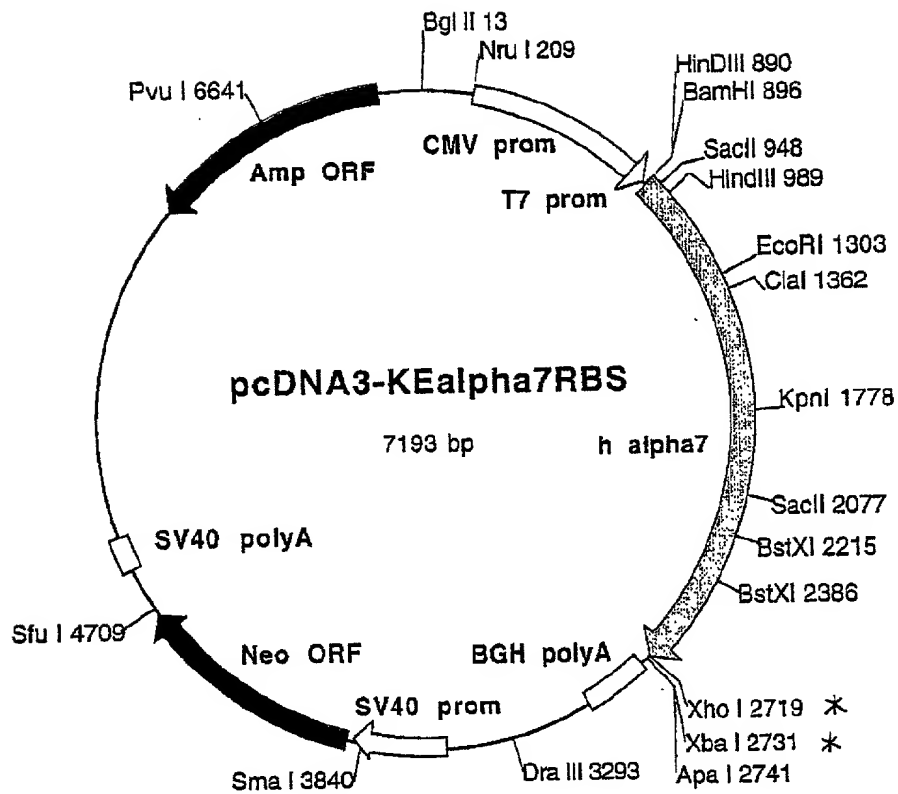
13. The isolated cell according to claim 6 wherein said cell expresses a functional neuronal nicotinic acetylcholine receptor that comprises a multimeric subunit combination, wherein said multimeric subunit combination is defined by the general formula  $\alpha_X\beta_2\beta_3, \beta_4$ , where X is one or more of  $\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5, \alpha_6$  or  $\alpha_7$ .

## Abstract

Isolated nucleic acid molecules, i.e., DNA or RNA encoding human neuronal nicotinic acetylcholine receptor alpha and beta subunits, mammalian and amphibian cells containing said DNA, methods for producing  $\alpha$  and  $\beta$  subunits and recombinant (i.e., isolated or substantially pure)  $\alpha$  subunits and  $\beta$  subunits are provided. In addition, cells expressing various multimeric combinations of subunits (i.e.,  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_6$  and/or  $\alpha_7$  in combination with at least one of an  $\alpha$  and  $\beta$  subunit are also provided. A recombinant, non-human cell line expressing the human  $\alpha_7$  subunit of nAChR is also disclosed.



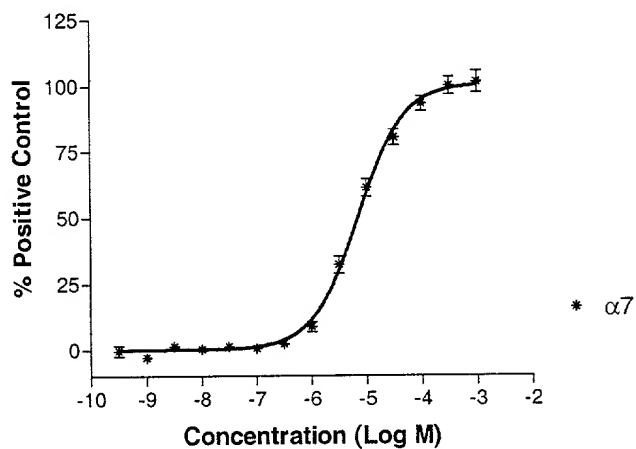
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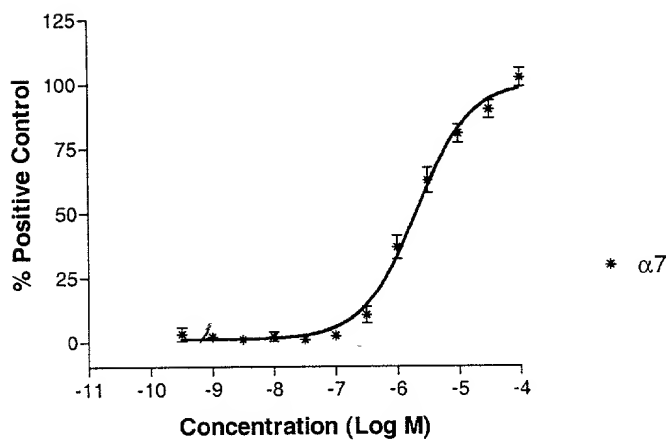
## Agonist-Induce Increases in $[Ca^{2+}]_i$ for A7 Stable Cell Line

(Expressing the Nicotinic Alpha 7 Receptor in GH<sub>4</sub>C<sub>1</sub> Cells)

### Acetylcholine

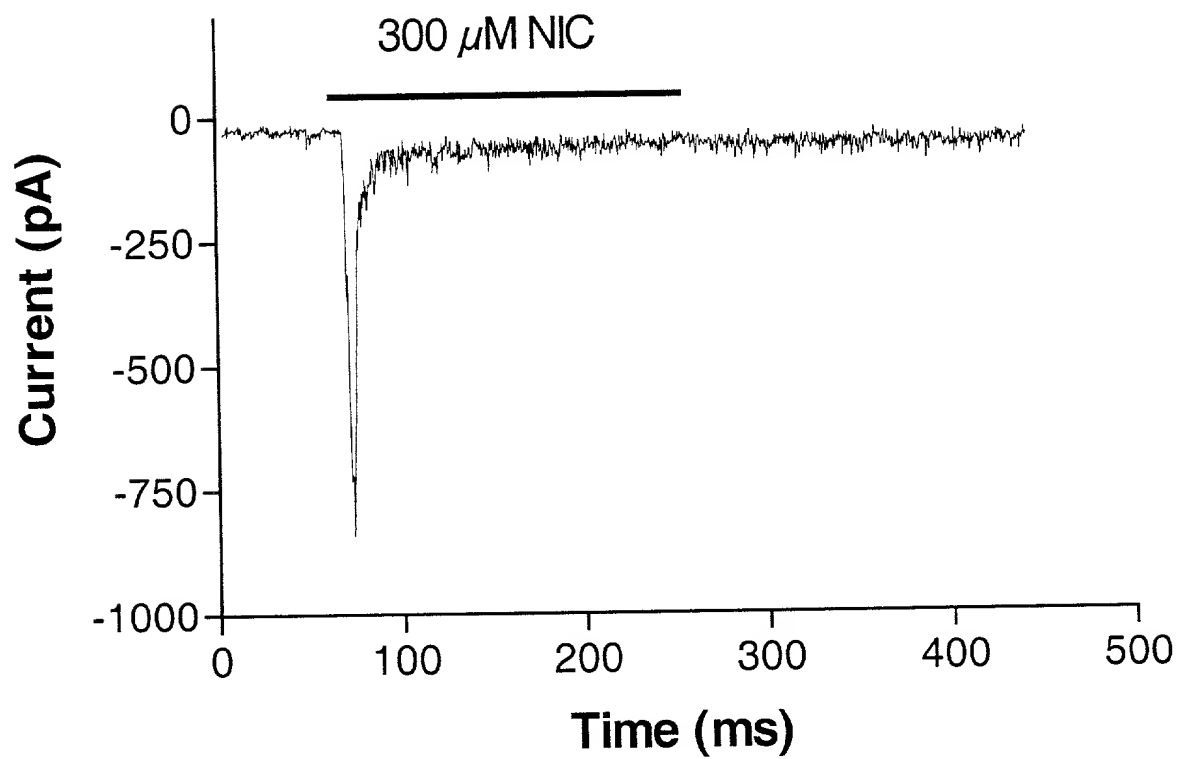


### Nicotine



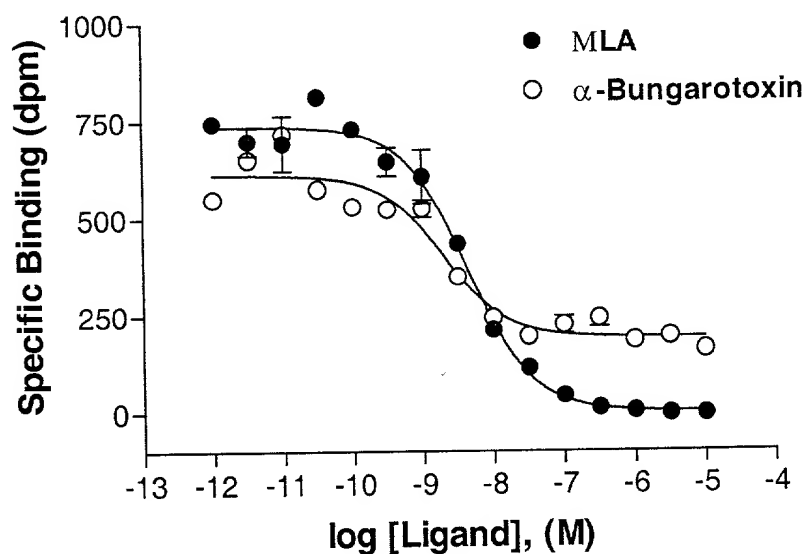
4

# G1-9-15-8 (A7) Cell





# **[<sup>3</sup>H]-Methyllycaconitine Binding to Membranes Prepared from $\alpha 7$ expressed in GH4C1 Whole Cells**



	MLA	$\alpha$ -Bungarotoxin
BOTTOM	0.3228	191.7
TOP	737.1	612.3
LOGEC50	-8.353	-8.689
HILLSLOPE	-0.9527	-0.9856
EC50	4.4320e-009	2.0470e-009

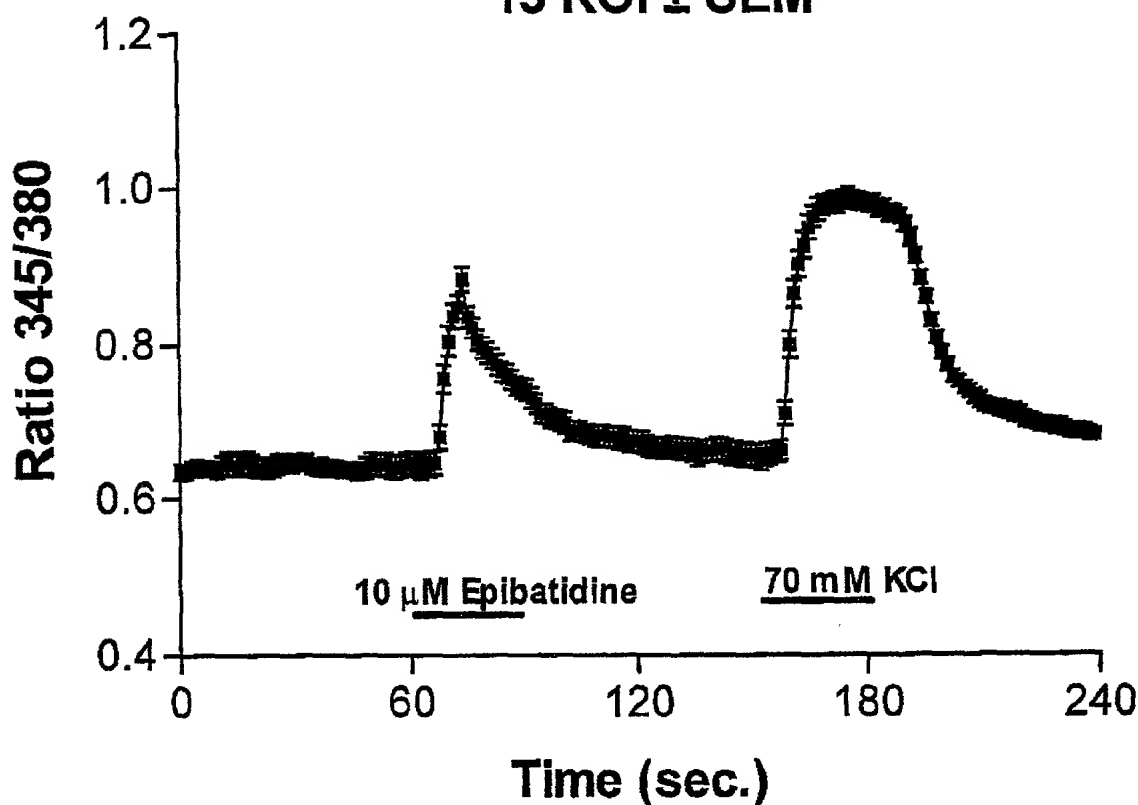
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91019ssdb

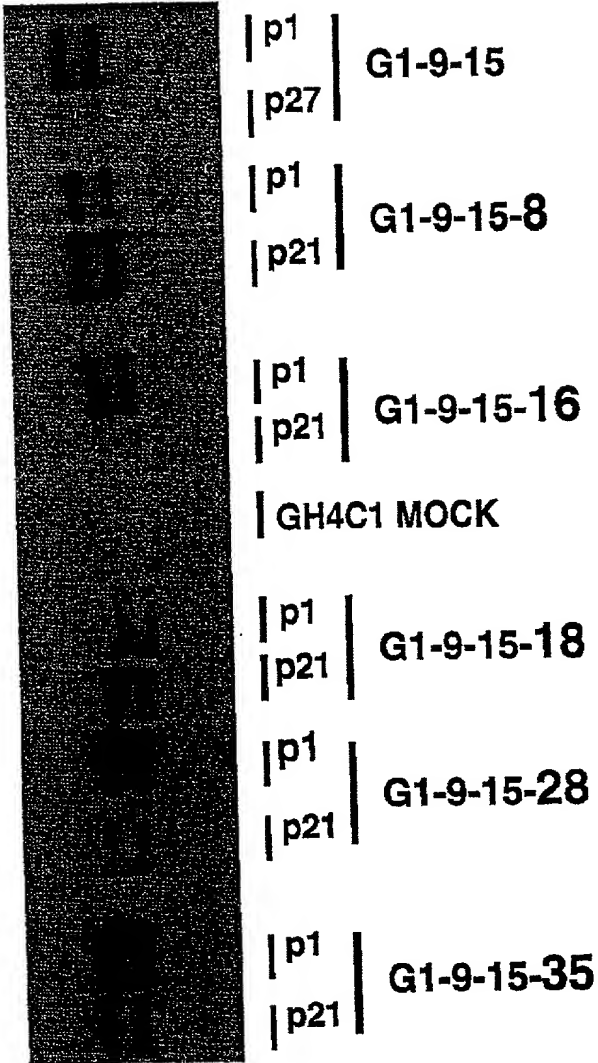
# Single-Cell Imaging Data Demonstrates the Homogeneous Response of Stable Cell Line A7 to Epibatidine

$\alpha_7$  Subclone G1-9-15-8  
15 ROI  $\pm$  SEM



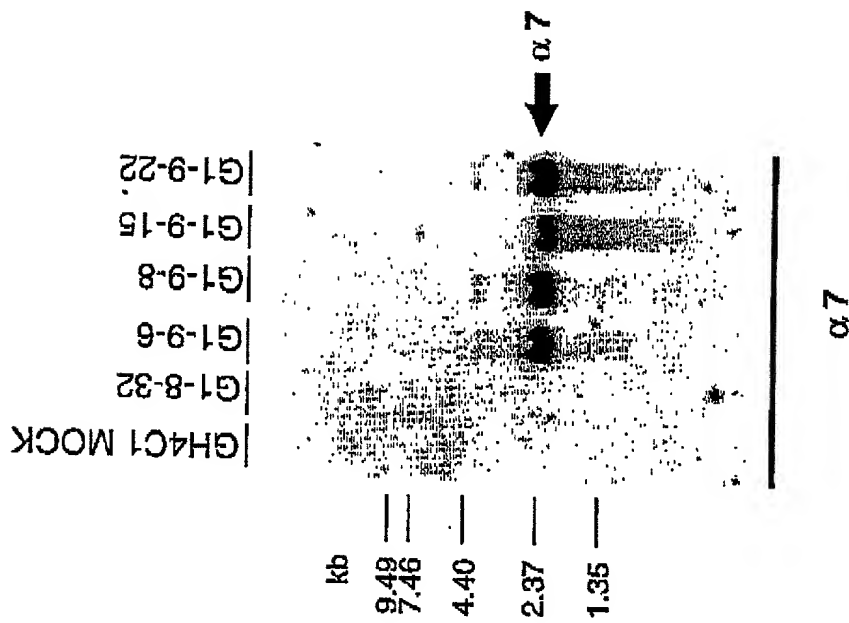
Cells were superfused with HBS prior to treatment periods as indicated on the graph. Values are means  $\pm$  SEM from one experiment. Cells were cultured at 37°C.

# G1-9-15 Subclones Stably Express $\alpha 7$ Protein



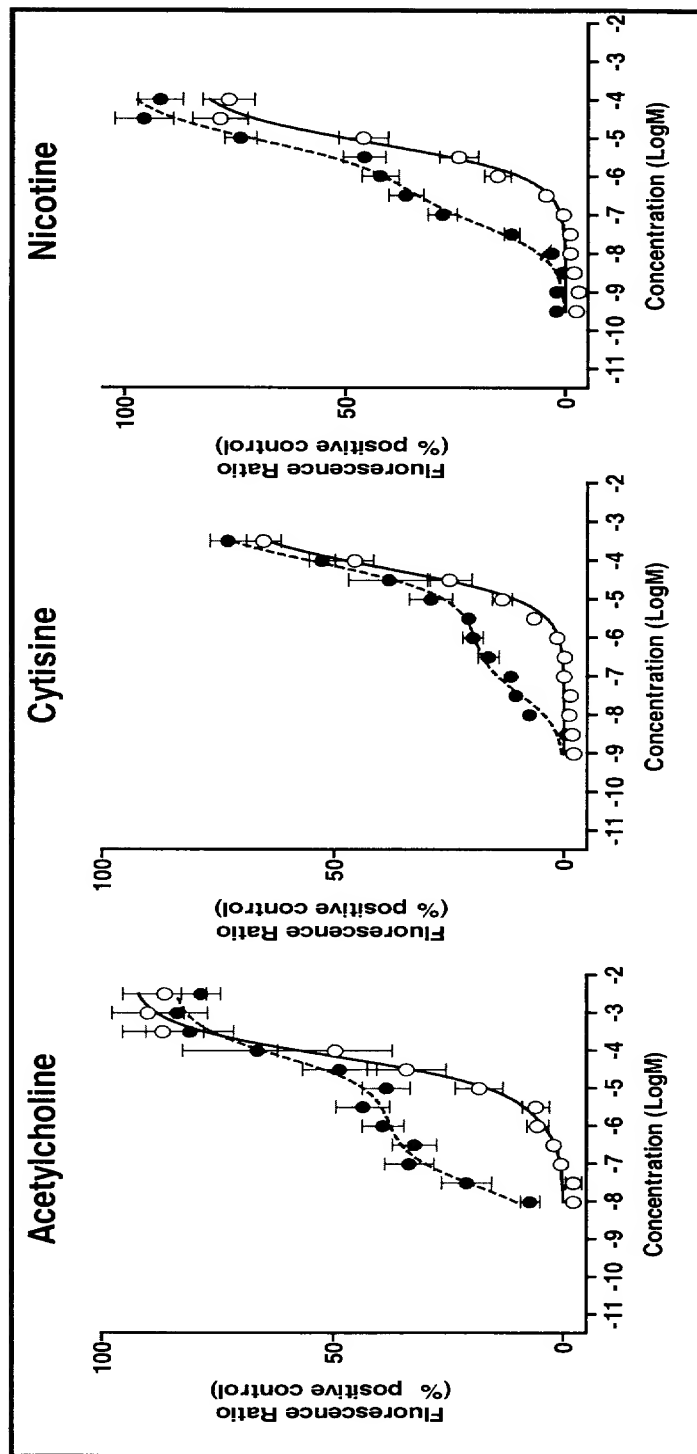
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9a CANNED, # 18

# Agonist Pharmacology of A3B2A5 Cells Is Distinct from A3B2 ( $\alpha 3\beta 2$ ) Cells

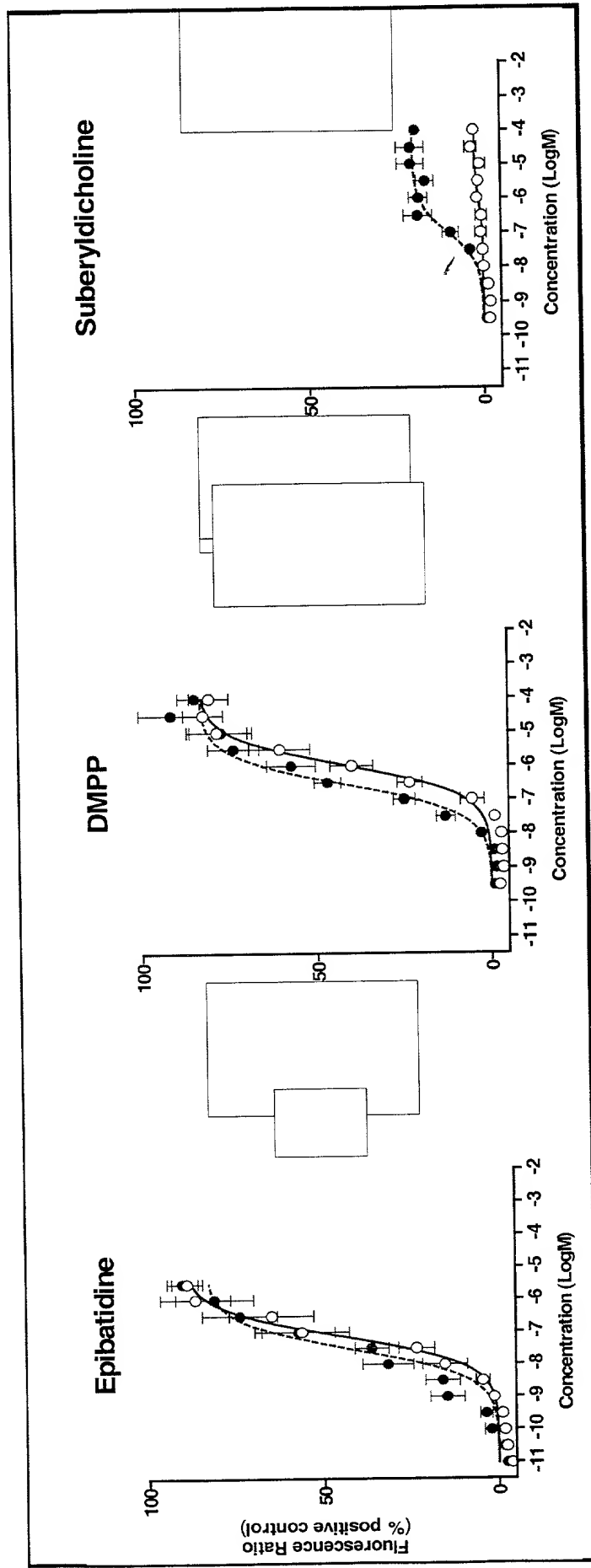


○ A3B2  
● A3B2A5  
5

\* Positive control Agonist is 30 $\mu$ M DMPP

CONFIDENTIAL

# Agonist Pharmacology of A3B2A5 Cells Is Distinct From A3B2 ( $\alpha 3\beta 2$ ) Cells



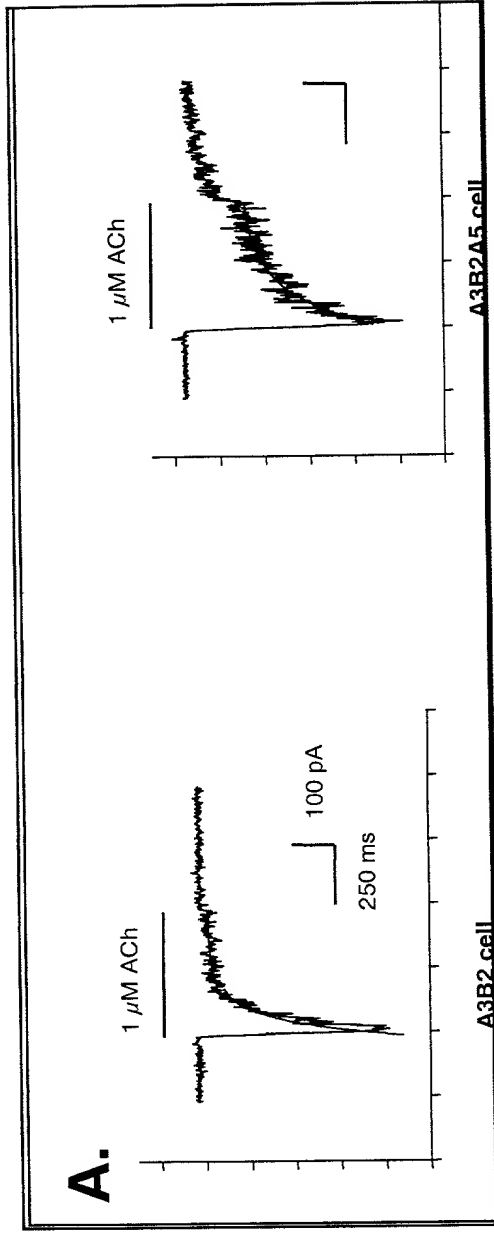
○ A3B2

● A3B2A

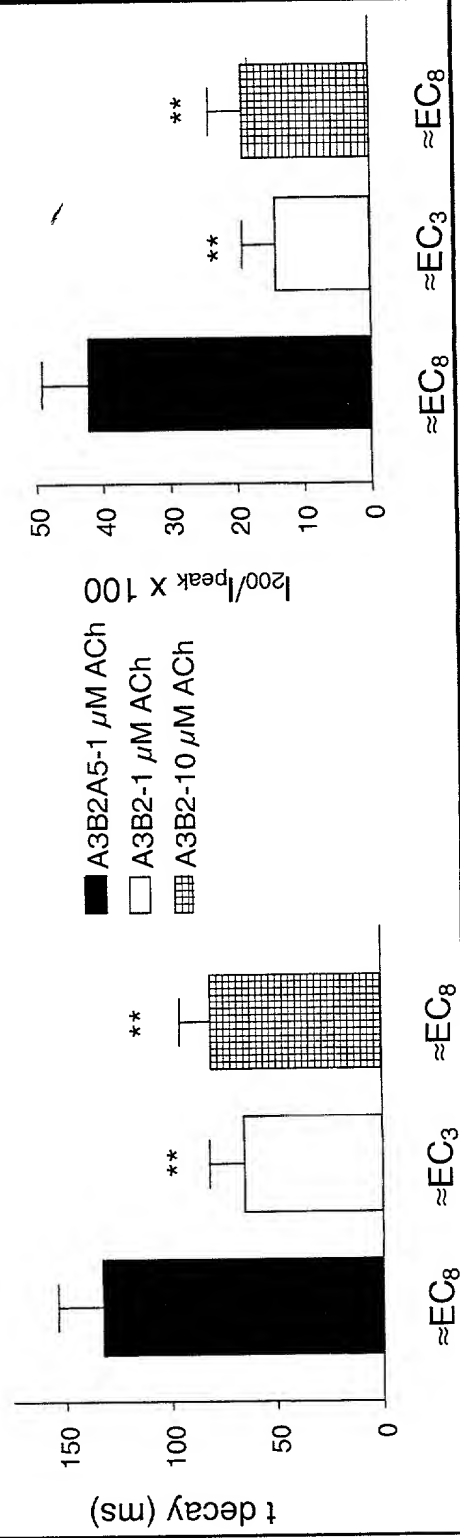
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\* Positive Control Agonist is 30 $\mu$ M DMPP

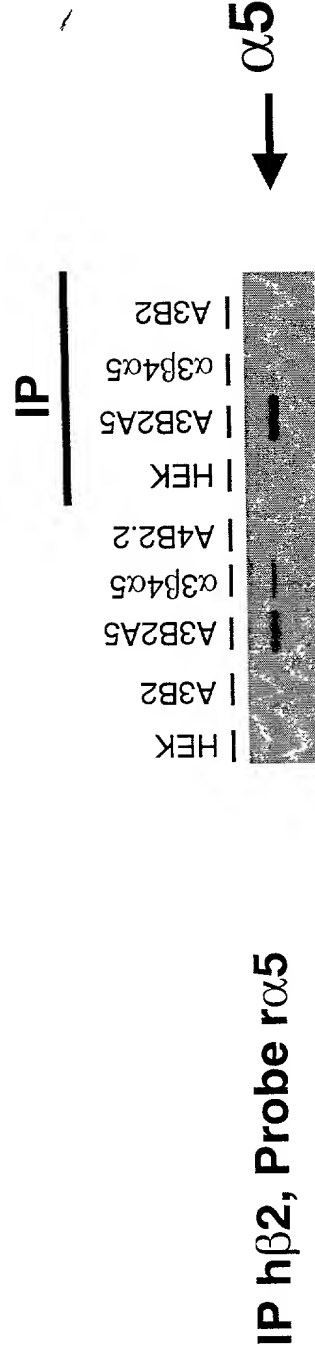
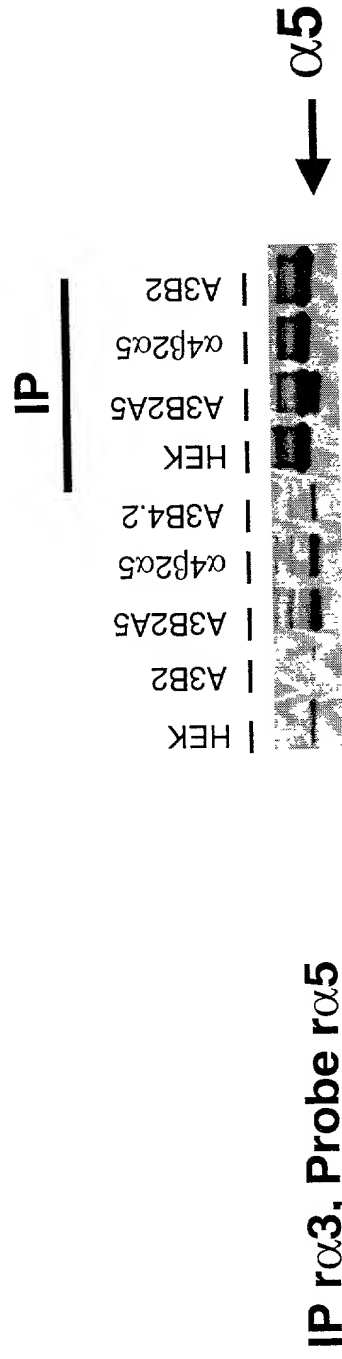
# The Kinetics of Decay of Currents Induced by Low Doses of Acetylcholine Are Slower in A3B2A5 than in A3B2 Cells



**B.** Time constant of decay of currents induced by ACh

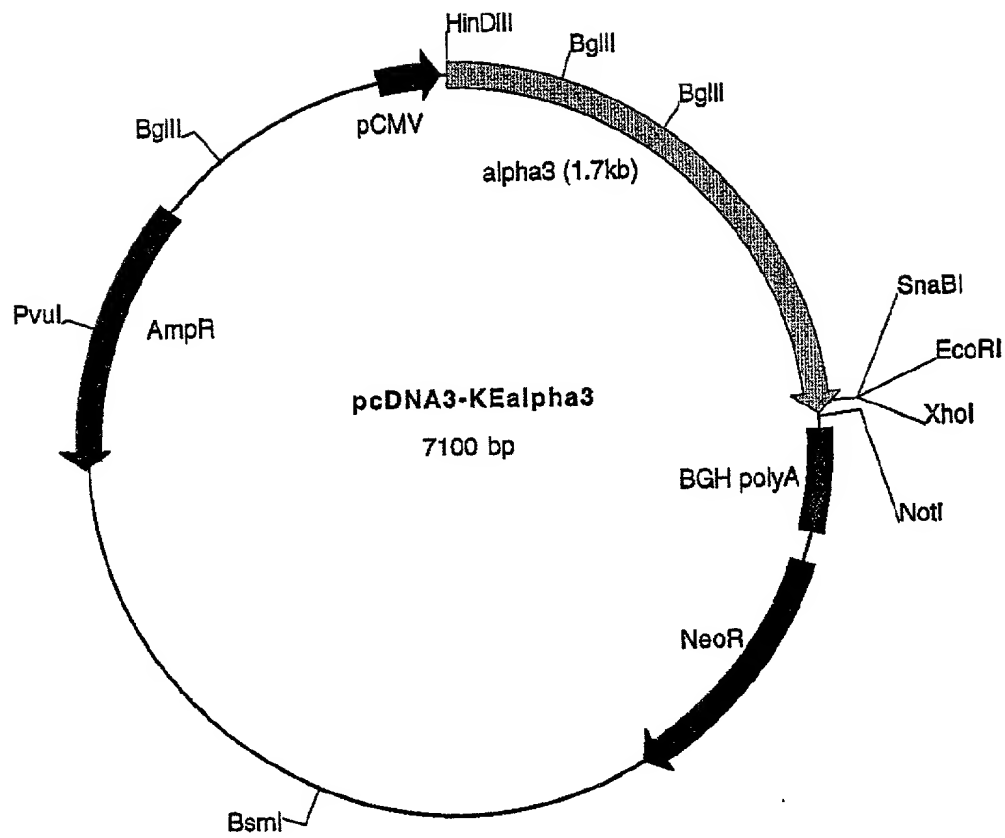


# $\alpha$ 5 Co-Assembles with $\alpha$ 3 and $\beta$ 2 in Cell Line A3B2A5

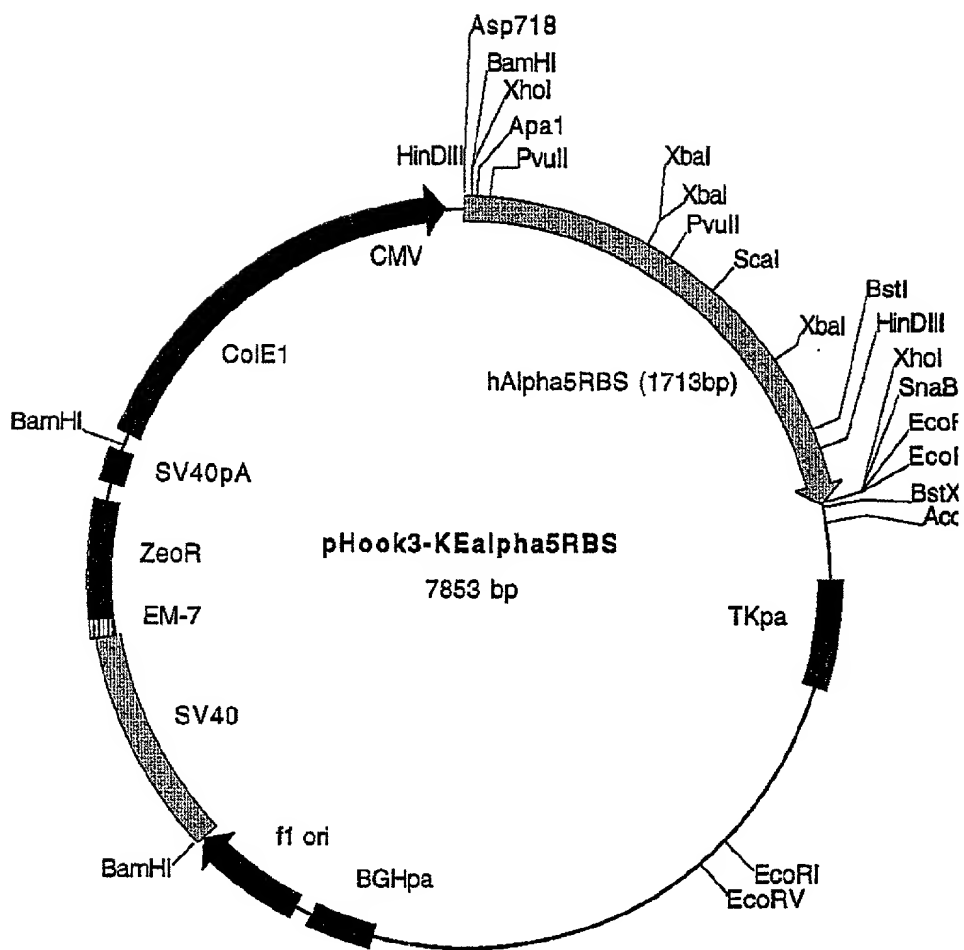




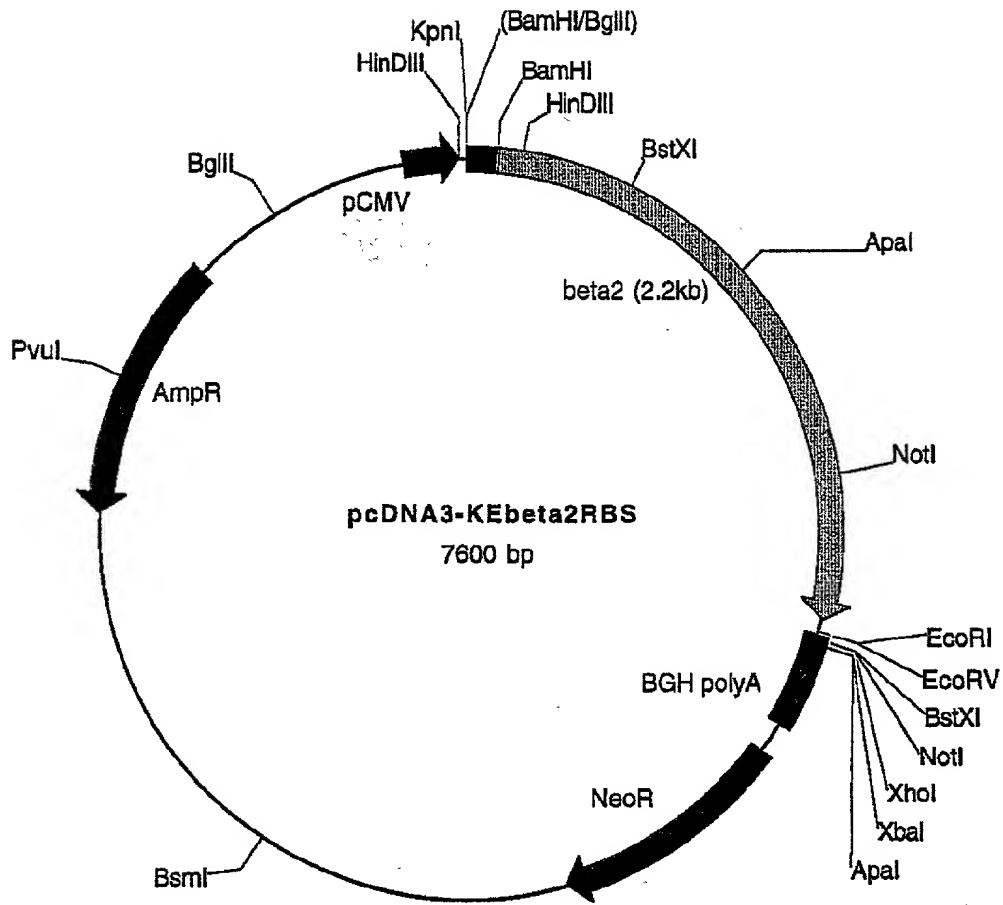
12



13



14



**DECLARATION AND  
POWER OF ATTORNEY  
FOR UTILITY OR DESIGN  
PATENT APPLICATION  
(37 CFR 1.63)**

☐ Declaration Submitted with Initial Filing **OR** ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number SD99511A

First Named Inventor A. GILLESPIE, ET AL.

**COMPLETE IF KNOWN**

Application Number UNKNOWN

Filing Date November 1, 2000

Group Art Unit PRIOR 1646

Examiner Name

**As a below named inventor, I hereby declare that:**

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

DNA ENCODING HUMAN  $\alpha$  AND  $\beta$  SUBUNITS OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR, CELLS TRANSFORMED THEREWITH, AND RECOMBINANT CELL LINE EXPRESSING A HUMAN  $\alpha$  AND  $\beta$  SUBUNIT OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR

(Title of the Invention)

the specification of which

☒ is attached hereto

OR

☐ was filed on (MM/DD/YYYY) as United States Application Number or PCT International

Application Number and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Attorney Docket Number	Priority Claimed?	
				YES	NO
				<input type="checkbox"/>	<input type="checkbox"/>
				<input type="checkbox"/>	<input type="checkbox"/>
				<input type="checkbox"/>	<input type="checkbox"/>
				<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	Attorney Docket Number
08/487,596	06/07/1995	SD-9951

**DECLARATION AND POWER OF ATTORNEY for Utility or Design Patent Application**

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known to me to be material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Application Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint, respectively and individually, as my attorneys or agents with full power of substitution and revocation, the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

☐ Customer Number  
OR

☒ Registered practitioner(s) name/registration number listed below

Place Customer Number  
Bar Code Label here

Name	Registration Number	Name	Registration Number
VINEET KOHLI	37,003		

Direct all correspondence to: ☒ Customer Number or Bar Code Label

000210

Name	VINEET KOHLI				
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Address	P.O. Box 2000, RY60-30				
City	Rahway	State	NJ	ZIP	07065-0907
Country	USA	Telephone	(732)594-3889	Fax	(732)594-4720

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])		Family Name or Surname			
ALISON		GILLESPIE			
Inventor's Signature				Date	
Residence: City	SAN DIEGO	State	CA	Country	US
				Citizenship	UK
Post Office Address	Merck & Co., Inc., P.O. Box 2000				
City	Rahway	State	NJ	ZIP	07065-0907

☒ Additional inventors are being named on the \_\_\_\_\_ supplemental Additional Inventors(s) sheet(s) PTO/SB/02A attached hereto.

## DECLARATION AND POWER OF ATTORNEY

ADDITIONAL INVENTOR(S)  
Supplemental Sheet

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])				Family Name or Surname					
BRIAN O.				CLAEPS					
Inventor's Signature						Date			
Residence: City		SAN DIEGO		State CA		Country US		Citizenship US	
Post Office Address		Merck & Co., Inc., P.O. Box 2000							
City		Rahway		State NJ		ZIP		07065-0907	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])				Family Name or Surname					
LAURA ELENA				CHAVEZ-NOVIEGA					
Inventor's Signature						Date			
Residence: City		SAN DIEGO		State CA		Country US		Citizenship MEXICO	
Post Office Address		Merck & Co., Inc., P.O. Box 2000							
City		Rahway		State NJ		ZIP		07065-0907	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])				Family Name or Surname					
ROBERT				SIEGEL					
Inventor's Signature						Date			
Residence: City		SAN DIEGO		State CA		Country US		Citizenship US	
Post Office Address		Merck & Co., Inc., P.O. Box 2000							
City		Rahway		State NJ		ZIP		07065-0907	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])				Family Name or Surname					
KATHRYN J.				ELLIOTT					
Inventor's Signature						Date			
Residence: City		SAN DIEGO		State CA		Country US		Citizenship US	
Post Office Address		Merck & Co., Inc., P.O. Box 2000							
City		Rahway		State NJ		ZIP		07065-0907	

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2277 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 166..1755
- (D) OTHER INFORMATION: /product= "ALPHA-2 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGCAACGTGG ATGCCGAGGA GAGGAGGTG GTGGTGGAGG AGGAGGACAG ATGGGCATGT	1440

GCAGGTCATG TGGCCCCCTC TGTGGGCACC CTCTGCAGCC ACGGCCACCT GCACTCTGGG 1500  
 GCCTCAGGTC CCAAGGCTGA GGCTCTGCTG CAGGAGGGTG AGCTGCTGCT ATCACCCAC 1560  
 ATGCAGAAGG CACTGGAAGG TGTGCACTAC ATTGCCGACC ACCTGCCGTC TGAGGATGCT 1620  
 GACTCTTCGG TGAAGGAGGA CTGGAAGTAT GTTGCCATGG TCATCGACAG GATCTTCCTC 1680  
 TGGCTGTTTA TCATCGTCTG CTTCCTGGGG ACCATCGGCC TCTTTCTGCC TCCGTTCTTA 1740  
 GCTGGAATGA TCTGACTGCA CCTCCCTCGA GCTGGCTCCC AGGGCAAAGG GGAGGGTTCT 1800  
 TGGATGTGGA AGGGCTTTGA ACAATGTTTA GATTTGGAGA TGAGCCCAA GTGCCAGGGA 1860  
 GAACAGCCAG GTGAGGTGGG AGGTTGGAGA GCCAGGTGAG GTCTCTCTAA GTCAGGCTGG 1920  
 GGTGAAGTT TGGAGTCTGT CCGAGTTTGC AGGGTGCTGA GCTGTATGGT CCAGCAGGGG 1980  
 AGTAATAAGG GCTCTTCCGG AAGGGGAGGA AGCGGGAGGC AGGGCCTGCA CCTGATGTGG 2040  
 AGGTACAGGG CAGATCTTCC CTACCGGGA GGGATGGATG GTTGGATACA GGTGGCTGGG 2100  
 CTATTCCATC CATCTGGAAG CACATTTGAG CCTCCAGGCT TCTCCTTGAC GTCATTCTCTC 2160  
 TCCTTCCTTG CTCCAAAATG GCTCTGCACC AGCCGGCCCC CAGGAGGTCT GGCAGAGCTG 2220  
 AGAGCCATGG CCTGCAGGGG CTCCATATGT CCCTACGCGT GCAGCAGGCA AACAAGA 2277

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 529 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Pro Ser Cys Pro Val Phe Leu Ser Phe Thr Lys Leu Ser Leu  
 1 5 10 15  
 Trp Trp Leu Leu Leu Thr Pro Ala Gly Gly Glu Glu Ala Lys Arg Pro  
 20 25 30  
 Pro Pro Arg Ala Pro Gly Asp Pro Leu Ser Ser Pro Ser Pro Thr Ala  
 35 40 45  
 Leu Pro Gln Gly Gly Ser His Thr Glu Thr Glu Asp Arg Leu Phe Lys  
 50 55 60  
 His Leu Phe Arg Gly Tyr Asn Arg Trp Ala Arg Pro Val Pro Asn Thr  
 65 70 75 80  
 Ser Asp Val Val Ile Val Arg Phe Gly Leu Ser Ile Ala Gln Leu Ile  
 85 90 95  
 Asp Val Asp Glu Lys Asn Gln Met Met Thr Thr Asn Val Trp Leu Lys  
 100 105 110  
 Gln Glu Trp Ser Asp Tyr Lys Leu Arg Trp Asn Pro Ala Asp Phe Gly  
 115 120 125  
 Asn Ile Thr Ser Leu Arg Val Pro Ser Glu Met Ile Trp Ile Pro Asp  
 130 135 140



Ile Val Leu Tyr Asn Asn Ala Asp Gly Glu Phe Ala Val Thr His Met  
 145 150 155 160  
 Thr Lys Ala His Leu Phe Ser Thr Gly Thr Val His Trp Val Pro Pro  
 165 170 175  
 Ala Ile Tyr Lys Ser Ser Cys Ser Ile Asp Val Thr Phe Phe Pro Phe  
 180 185 190  
 Asp Gln Gln Asn Cys Lys Met Lys Phe Gly Ser Trp Thr Tyr Asp Lys  
 195 200 205  
 Ala Lys Ile Asp Leu Glu Gln Met Glu Gln Thr Val Asp Leu Lys Asp  
 210 215 220  
 Tyr Trp Glu Ser Gly Glu Trp Ala Ile Val Asn Ala Thr Gly Thr Tyr  
 225 230 235 240  
 Asn Ser Lys Lys Tyr Asp Cys Cys Ala Glu Ile Tyr Pro Asp Val Thr  
 245 250 255  
 Tyr Ala Phe Val Ile Arg Arg Leu Pro Leu Phe Tyr Thr Ile Asn Leu  
 260 265 270  
 Ile Ile Pro Cys Leu Leu Ile Ser Cys Leu Thr Val Leu Val Phe Tyr  
 275 280 285  
 Leu Pro Ser Asp Cys Gly Glu Lys Ile Thr Leu Cys Ile Ser Val Leu  
 290 295 300  
 Leu Ser Leu Thr Val Phe Leu Leu Leu Ile Thr Glu Ile Ile Pro Ser  
 305 310 315 320  
 Thr Ser Leu Val Ile Pro Leu Ile Gly Glu Tyr Leu Leu Phe Thr Met  
 325 330 335  
 Ile Phe Val Thr Leu Ser Ile Val Ile Thr Val Phe Val Leu Asn Val  
 340 345 350  
 His His Arg Ser Pro Ser Thr His Thr Met Pro His Trp Val Arg Gly  
 355 360 365  
 Ala Leu Leu Gly Cys Val Pro Arg Trp Leu Leu Met Asn Arg Pro Pro  
 370 375 380  
 Pro Pro Val Glu Leu Cys His Pro Leu Arg Leu Lys Leu Ser Pro Ser  
 385 390 395 400  
 Tyr His Trp Leu Glu Ser Asn Val Asp Ala Glu Glu Arg Glu Val Val  
 405 410 415  
 Val Glu Glu Glu Asp Arg Trp Ala Cys Ala Gly His Val Ala Pro Ser  
 420 425 430  
 Val Gly Thr Leu Cys Ser His Gly His Leu His Ser Gly Ala Ser Gly  
 435 440 445  
 Pro Lys Ala Glu Ala Leu Leu Gln Glu Gly Glu Leu Leu Leu Ser Pro  
 450 455 460  
 His Met Gln Lys Ala Leu Glu Gly Val His Tyr Ile Ala Asp His Leu  
 465 470 475 480  
 Arg Ser Glu Asp Ala Asp Ser Ser Val Lys Glu Asp Trp Lys Tyr Val  
 485 490 495

Ala Met Val Ile Asp Arg Ile Phe Leu Trp Leu Phe Ile Ile Val Cys  
500 505 510  
Phe Leu Gly Thr Ile Gly Leu Phe Leu Pro Pro Phe Leu Ala Gly Met  
515 520 525  
Ile

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1757 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 39..1553  
(D) OTHER INFORMATION: /product= "ALPHA-3 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCGACCGTCC GGGTCGCGG CCAGCCCGGC CACCAGCCAT GGGCTCTGGC CCGCTCTCGC	60
TGCCCCCTGGC GCTGTCGCCG CCGCGGCTGC TGCTGCTGCT GCTGTCTCTG CTGCCAGTGG	120
CCAGGGCCTC AGAGGCTGAG CACCGTCTAT TTGAGCGGCT GTTTGAAGAT TACAATGAGA	180
TCATCCGGCC TGTAGCCAAC GTGTCTGACC CAGTCATCAT CCATTTCGAG GTGTCCATGT	240
CTCAGCTGGT GAAGGTGGAT GAAGTAAACC AGATCATGGA GACCAACCTG TGGCTCAAGC	300
AAATCTGGAA TGA CTACTACAAG CTGAAGTGA ACCCTCTGA CTATGGTGGG GCAGAGTTCA	360
TGCGTGTCCC TGCACAGAAG ATCTGGAAGC CAGACATTGT GCTGTATAAC AATGCTGTTG	420
GGGATTTCCA GGTGGACGAC AAGACCAAAG CCTTACTCAA GTACACTGGG GAGGTGACTT	480
GGATACCTCC GGCCATCTTT AAGAGCTCCT GTAAAATCGA CGTGACCTAC TTCCCGTTTG	540
ATTACCAAAA CTGTACCATG AAGTTCGGTT CCTGGTCCTA CGATAAGGCG AAAATCGATC	600
TGGTCCTGAT CGGCTCTTCC ATGAACCTCA AGGACTATTG GGAGAGCGGC GAGTGGGCCA	660
TCATCAAAGC CCCAGGCTAC AAACACGACA TCAAGTACAG CTGCTCCGAG GAGATCTACC	720
CCGACATCAC ATACTCGCTG WWCATCCGGC GGCTGTCGTT GTTCTACACC ATCAWCCTCA	780
TCATCCGCTG GCTGATCATC TCCTTCATCA CTGTGGTGGT CTCCTACCTG CCCTCCGACT	840
GCGGCGAGAA GGTGACCCTG TGYATTTCTG TCCTCCTCTC CCTGACGGTG TTTCTCCTGG	900
TGATCACTGA GACCATCCCT TCCACCTCGC TGGTCATCCC CCTGATTGGA GAGTACCTCC	960
TGWWCACCAT GATTTGTGTA ACCTGTCCA TCGACATCAC CGTCTGCGTG CTCAACGTGC	1020
ACTACAGAAC CCCGACGACA CACACAATGC CCTCATGGGT GAAGACTGTA TTCTTGAMCC	1080
TGCTCCCCAG GGTATGTWC ATGACCAGGC CAACAAGCAA CGAGGGCAAC GCTCAGPAGC	1140
CGAGGCCCTT CTACGGTGCC GAGCTCTCAA ATCTGAATTG CTTGAGCCGC GCAGAGTCCA	1200

AAGGCTGCAA GGAGGGCTAC CCCTGCCAGG ACGGGATGTG TGGTTACTGC CACCACCGCA 1260  
 GGATAAAAAT CTCCAATTTT AGTGCTAACC TCACGAGAAG CTCTAGTTCT GAATCTGTTG 1320  
 ATGCTGTGCT GTCCCTCTCT GCTTTGTCAC CAGAAATCAA AGAAGCCATC CAAAGTGTCA 1380  
 AGTATATTGC TGAAAATATG AAAGCACAAA ATGAAGCCAA AGAGATTCAA GATGATTGGA 1440  
 AGTATGTGTC CATGGTGATT GATCGTATTT TTCTGTGGGT TTTCACCCCTG GTGTGCATTG 1500  
 TAGGGACAGC AGGATTGTTT CTGCAACCCC TGATGGCCAG GGAAGATGCA TAAGCACTAA 1560  
 GCTGTGTGCC TGCCCTGGGAG ACTTCCTTGT GTCAGGGCAG GAGGAGGCTG CTTCCCTAGTA 1620  
 AGAACGTACT TTCTGTTATC AAGCTACCAG CTTT Tyr Asp Lys Ala Lys Ile Asp Leu Val Leu  
 Ile Gly

180

185

190

Ser Ser Met Asn Leu Lys Asp Tyr Trp Glu Ser Gly Glu Trp Ala Ile  
 195 200 205

Ile Lys Ala Pro Gly Tyr Lys His Asp Ile Lys Tyr Ser Cys Cys Glu  
 210 215 220

Glu Ile Tyr Pro Asp Ile Thr Tyr Ser Leu Xaa Ile Arg Arg Leu Ser  
 225 230 235 240

Leu Phe Tyr Thr Ile Xaa Leu Ile Ile Arg Trp Leu Ile Ile Ser Phe  
 245 250 255

Ile Thr Val Val Val Ser Tyr Leu Pro Ser Asp Cys Gly Glu Lys Val  
 260 265 270

Thr Leu Cys Ile Ser Val Leu Leu Ser Leu Thr Val Phe Leu Leu Val  
 275 280 285

Ile Thr Glu Thr Ile Pro Ser Thr Ser Leu Val Ile Pro Leu Ile Gly  
 290 295 300

Glu Tyr Leu Leu Xaa Thr Met Ile Cys Val Thr Leu Ser Ile Asp Ile  
 305 310 315 320

Thr Val Cys Val Leu Asn Val His Tyr Arg Thr Pro Thr Thr His Thr  
 325 330 335

Met Pro Ser Trp Val Lys Thr Val Phe Leu Xaa Leu Leu Pro Arg Val  
 340 345 350

Met Xaa Met Thr Arg Pro Thr Ser Asn Glu Gly Asn Ala Gln Lys Pro  
 355 360 365

Arg Pro Leu Tyr Gly Ala Glu Leu Ser Asn Leu Asn Cys Phe Ser Arg  
 370 375 380

Ala Glu Ser Lys Gly Cys Lys Glu Gly Tyr Pro Cys Gln Asp Gly Met  
 385 390 395 400

Cys Gly Tyr Cys His His Arg Arg Ile Lys Ile Ser Asn Phe Ser Ala  
 405 410 415

Asn Leu Thr Arg Ser Ser Ser Ser Glu Ser Val Asp Ala Val Leu Ser  
 420 425 430

Leu Ser Ala Leu Ser Pro Glu Ile Lys Glu Ala Ile Gln Ser Val Lys  
 435 440 445

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 380  
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Tyr Ile Ala Glu Asn Met Lys Ala Gln Asn Glu Ala Lys Glu Ile Gln  
 450 455 460  
 Asp Asp Trp Lys Tyr Val Ala Met Val Ile Asp Arg Ile Phe Leu Trp  
 465 470 475 480  
 Val Phe Thr Leu Val Cys Ile Leu Gly Thr Ala Gly Leu Phe Leu Gln  
 485 490 495  
 Pro Leu Met Ala Arg Glu Asp Ala  
 500

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2363 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 173..2056
- (D) OTHER INFORMATION: /product= "ALPHA-4 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGCTCGCTG CGGCGCCGCC GCCGCNCCGC GCGCCACAGG AGAAGGCGAN CCGGGCCCCG	60
CGGCCGAAGC GGCCCGCGAG GCGCGGGAGG CATGAAGTTG GCGCGGCACG GGCCTCGAAG	120
CGGCGGGGAG CCGGAGCCG CCCGCATCTA GAGCCCGCGA GGTGCGTGCG CCATGGAGCT	180
AGGGGGCCCC GGAGCGCCGC GGCTGCTGCC GCGCTGCTG CTGCTTCTGG GGACCGGCCT	240
CCTGCGCGCC AGCAGCCATG TGGAGACCCG GGGCCACGCC GAGGAGCGGC TCCTGAAGAA	300
ACTCTTCTCC GGTACAACA AGTGGTCCCG ACCCGTGGCC AACATCTCGG ACGTGGTCCT	360
CGTCCGCTTC GGCTGTCCA TCGCTCAGCT CATGACGTG GATGAGAAGA ACCAGATGAT	420
GACCACGAAC GTCTGGGTGA AGCAGGAGTG GCACGACTAC AAGCTGCGCT GGGACCCAGC	480
TGACTATGAG AATGTCACCT CCATCCGCAT CCCCTCCGAG CTCATCTGGC GGCCGGACAT	540
CGCCCTCTAC AACAAATGCTG ACGGGGACTT CGCGGCCACC CACCTGACCA AGGCCACCT	600
GTTCCATGAC GGGCGGGTGC AGCGGACTCC CCGGCCCAT TACAAGAGCT CCTGCAGCAT	660
CGACGTACCC TTCTTCCCCT TCGACCAGCA GAACTGCACC ATGAAATTGG GTCCTGGAC	720
CTACGACAAG GCCAAGATCG ACCTGGTGAA CATGCACAGC CGCGTGGACC AGCTGGACTT	780
CTGGGAGAGT GCGGAGTGGC TCATCTCGGA CGCGTGGGC ACCTACAACA CCAGGAAGTA	840
CGAGTGCTGC GCGAGATCT ACCCGGACAT CACCTATGCC TACGCCATCC GCGCGCTGCC	900
GCTCTTCTAC ACCATCAACT TCATCATCCC CTGGCTGCTC ATCTCCTGCC TCACCGCGCT	960
GGTCTTCTAC CTGCCCTCCG AGTGTGGCGA GAAGATCAGC CTGTGCATCT CCGTGTGCT	1020
GTGCGTCACC GTCTTCTGCT TGCTCATCAC CGAGATCATC CCGTCCACCT CACTGGTCAT	1080

CCCACTCATC GGCAGTACC TGCTGTTCAC CATGATCTTC GTCACCCTGT CCATCGCCAT	1140
CACGGTCTTC GTGCTCAACG TGCACCACCG CTCGCCACGC ACGCACACCA TGCCACCTG	1200
GGTACGCAGG GTCTTCCTGG ACATCGTGCC ACGCCTGCTC CTCATGAAGC GGCCGTCCGT	1260
GGTCAAGGAC AATTGCCGGC GGCTCATCGA GTCCATGCAT AAGATGGCCA GTGCCCCGCG	1320
CTTCTGGCCC GAGCCAGAAG GGGAGCCCCC TGCCACGAGC GGCACCCAGA GCCTGCACCC	1380
TCCCTCACCG TCCTTCTGCG TCCCTCTGGA TGTGCCGGCT GAGCCTGGGC CTTCCTGCAA	1440
GTCACCCTCC GACCAGCTCC CTCCTCAGCA GCCCCTGGAA GCTGAGAAAG CCAGCCCCCA	1500
CCCTTCGGCT GGACCTGCC GCGCGCCCCA CGGCACCCAG GCACCAGGC TGGCCAAAGC	1560
CAGGTCCCTC AGCGTCCAGC ACATGTCCAG CCCTGGCGAA GCGGTGGAAG GCGGCTCCG	1620
GTGCCGGTCT CGGAGCATCC AGTACTGTGT TCCCGGAGC GATGCCGCC CCGAGGCAGA	1680
TGCCAGGCT GCGGCGCCCC TGGCTCTCG CAACAGCCAC TCGGCTGAGC TCCACCCCC	1740
AGACCAGCCC TCTCCGTGCA AATGCACATG CAAGAAGGAG CCCTCTTCGG TGTCCTCCGAG	1800
CGCCACGGTC AAGACCGCA GCACCAAAGC GCGCGCGCG CACCTGCCCC TGTGCGCGGC	1860
CCTGAGCCGG GCGGTGGAGG GCGTCCAGTA CATTGCAGAC CACCTGAAG CCGAGACAC	1920
AGACTTCTCG GTGAAGGAG ACTGGAAGTA CGTGCCCATG GTCATCGACC GCATCTTCCT	1980
CTGGATGTTT ATCATCGTCT GCCTGCTGGG GACGGTGGGC CTCTTCCTGC CGCCCTGGCT	2040
GGCTGGCATG ATCTAGGAAG GGACCGGGAG CCTGCGTGGC CTGGGGCTGC CGYGCACGGG	2100
GCCAGCATCC ATGCGGCCGG CCTGGGGCCG GGCTGGCTTC TCCCTGGACT CTGTGGGGCC	2160
ACACGTTTGC CAAATTTTCC TTCCTGTCTT GTGTCTGCTG TAAGACGGCC TTGGACGGCG	2220
ACACGGCCTC TGGGGAGACC GAGTGTGGAG CTGCTTCCAG TTGGACTCTS GCCTCAGNAG	2280
GCAGCGGCTT GGAGCAGAGG TGGCGGTGCG CGCCTYCTAC CTGCAGGACT CGGGCTAAGT	2340
CCAGCTCTCC CCCTGCGCAG CCC	2363

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 627 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Glu	Leu	Gly	Gly	Pro	Gly	Ala	Pro	Arg	Leu	Leu	Pro	Pro	Leu	Leu
1				5				10						15	
Leu	Leu	Leu	Gly	Thr	Gly	Leu	Leu	Arg	Ala	Ser	Ser	His	Val	Glu	Thr
			20					25					30		
Arg	Ala	His	Ala	Glu	Glu	Arg	Leu	Leu	Lys	Lys	Leu	Phe	Ser	Gly	Tyr
			35				40					45			
Asn	Lys	Trp	Ser	Arg	Pro	Val	Ala	Asn	Ile	Ser	Asp	Val	Val	Leu	Val
			50			55					60				

Arg Phe Gly Leu Ser Ile Ala Gln Leu Ile Asp Val Asp Glu Lys Asn  
 65 70 75 80  
 Gln Met Met Thr Thr Asn Val Trp Val Lys Gln Glu Trp His Asp Tyr  
 85 90 95  
 Lys Leu Arg Trp Asp Pro Ala Asp Tyr Glu Asn Val Thr Ser Ile Arg  
 100 105 110  
 Ile Pro Ser Glu Leu Ile Trp Arg Pro Asp Ile Ala Leu Tyr Asn Asn  
 115 120 125  
 Ala Asp Gly Asp Phe Ala Ala Thr His Leu Thr Lys Ala His Leu Phe  
 130 135 140  
 His Asp Gly Arg Val Gln Arg Thr Pro Pro Ala Ile Tyr Lys Ser Ser  
 145 150 155 160  
 Cys Ser Ile Asp Val Thr Phe Phe Pro Phe Asp Gln Gln Asn Cys Thr  
 165 170 175  
 Met Lys Phe Gly Ser Trp Thr Tyr Asp Lys Ala Lys Ile Asp Leu Val  
 180 185 190  
 Asn Met His Ser Arg Val Asp Gln Leu Asp Phe Trp Glu Ser Gly Glu  
 195 200 205  
 Trp Leu Ile Ser Asp Ala Val Gly Thr Tyr Asn Thr Arg Lys Tyr Glu  
 210 215 220  
 Cys Cys Ala Glu Ile Tyr Pro Asp Ile Thr Tyr Ala Tyr Ala Ile Arg  
 225 230 235 240  
 Arg Leu Pro Leu Phe Tyr Thr Ile Asn Leu Ile Ile Pro Trp Leu Leu  
 245 250 255  
 Ile Ser Cys Leu Thr Ala Leu Val Phe Tyr Leu Pro Ser Glu Cys Gly  
 260 265 270  
 Glu Lys Ile Thr Leu Cys Ile Ser Val Leu Leu Ser Leu Thr Val Phe  
 275 280 285  
 Leu Leu Leu Ile Thr Glu Ile Ile Pro Ser Thr Ser Leu Val Ile Pro  
 290 295 300  
 Leu Ile Gly Glu Tyr Leu Leu Phe Thr Met Ile Phe Val Thr Leu Ser  
 305 310 315 320  
 Ile Ala Ile Thr Val Phe Val Leu Asn Val His His Arg Ser Pro Arg  
 325 330 335  
 Thr His Thr Met Pro Thr Trp Val Arg Arg Val Phe Leu Asp Ile Val  
 340 345 350  
 Pro Arg Leu Leu Leu Met Lys Arg Pro Ser Val Val Lys Asp Asn Cys  
 355 360 365  
 Arg Arg Leu Ile Glu Ser Met His Lys Met Ala Ser Ala Pro Arg Phe  
 370 375 380  
 Trp Pro Glu Pro Glu Gly Glu Pro Pro Ala Thr Ser Gly Thr Gln Ser  
 385 390 395 400  
 Leu His Pro Pro Ser Pro Ser Phe Cys Val Pro Leu Asp Val Pro Ala  
 405 410 415

Glu Pro Gly Pro Ser Cys Lys Ser Pro Ser Asp Gln Leu Pro Pro Gln  
 420 425 430  
 Gln Pro Leu Glu Ala Glu Lys Ala Ser Pro His Pro Ser Pro Gly Pro  
 435 440 445  
 Cys Arg Pro Pro His Gly Thr Gln Ala Pro Gly Leu Ala Lys Ala Arg  
 450 455 460  
 Ser Leu Ser Val Gln His Met Ser Ser Pro Gly Glu Ala Val Glu Gly  
 465 470 475 480  
 Gly Val Arg Cys Arg Ser Arg Ser Ile Gln Tyr Cys Val Pro Arg Asp  
 485 490 495  
 Asp Ala Ala Pro Glu Ala Asp Gly Gln Ala Ala Gly Ala Leu Ala Ser  
 500 505 510  
 Arg Asn Ser His Ser Ala Glu Leu Pro Pro Pro Asp Gln Pro Ser Pro  
 515 520 525  
 Cys Lys Cys Thr Cys Lys Lys Glu Pro Ser Ser Val Ser Pro Ser Ala  
 530 535 540  
 Thr Val Lys Thr Arg Ser Thr Lys Ala Pro Pro Pro His Leu Pro Leu  
 545 550 555 560  
 Ser Pro Ala Leu Ser Arg Ala Val Glu Gly Val Gln Tyr Ile Ala Asp  
 565 570 575  
 His Leu Lys Ala Glu Asp Thr Asp Phe Ser Val Lys Glu Asp Trp Lys  
 580 585 590  
 Tyr Val Ala Met Val Ile Asp Arg Ile Phe Leu Trp Met Phe Ile Ile  
 595 600 605  
 Val Cys Leu Leu Gly Thr Val Gly Leu Phe Leu Pro Pro Trp Leu Ala  
 610 615 620  
 Gly Met Ile  
 625

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1828 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 155..1561
- (D) OTHER INFORMATION: /product= "ALPHA-5 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCGGCGGGA GCTGTGGCGC GGAGCGGCCG CGCTGCTGCG TCTGCCCTCG TTTTGTCTCA	60
CGACTCACAC TCAGTGCTGC ATTCCCAAG AGTTGCGGTT CCCCAGCGGG CGGTGAGAG	120
GCGGCTGCCC GCGGTCCGCG CCGGGCGCGG GGCG ATG GCG GCG CGG GGG TCA	172
Met Ala Ala Arg Gly Ser	
1 5	

GGG CCC CGC GCG CTC CGC CTG CTG CTC TTG GTC CAG CTG GTC GCG GGG Gly Pro Arg Ala Leu Arg Leu Leu Val Gln Leu Val Ala Gly 10 15 20	220
CGC TGC GGT CTA GCG GGC GCG GCG GGC GCG CAG AGA GGA TTA TCT Arg Cys Gly Leu Ala Gly Ala His Gly Gly Ala Gln Arg Gly Leu Ser 25 30 35	268
GAA CCT TCT TCT ATT GCA AAA CAT GAA GAT AGT TTG CTT AAG GAT TTA Glu Pro Ser Ser Ile Ala Lys His Glu Asp Ser Leu Leu Lys Asp Leu 40 45 50	316
TTT CAA GAC TAC GAA AGA TGG GTT CGT CCT GTG GAA CAC CTG AAT GAC Phe Gln Asp Tyr Glu Arg Trp Val Arg Pro Val Glu His Leu Asn Asp 55 60 65 70	364
AAA ATA AAA ATA AAA TTT GGA CTT GCA ATA TCT CAA TTG GTG GAT GTG Lys Ile Lys Ile Lys Phe Gly Leu Ala Ile Ser Gln Leu Val Asp Val 75 80 85	412
GAT GAG AAA AAT CAG TTA ATG ACA ACA AAC GTC TGG TTG AAA CAG GAA Asp Glu Lys Asn Gln Leu Met Thr Thr Asn Val Trp Leu Lys Gln Glu 90 95 100	460
TGG ATA GAT GTA AAA TTA AGA TGG AAC CCT GAT GAC TAT GGT GGA ATA Trp Ile Asp Val Lys Leu Arg Trp Asn Pro Asp Asp Tyr Gly Gly Ile 105 110 115	508
AAA GTT ATA CGT GTT CCT TCA GAC TCT GTC TGG ACA CCA GAC ATC GTT Lys Val Ile Arg Val Pro Ser Asp Ser Val Trp Thr Pro Asp Ile Val 120 125 130	556
TTG TTT GAT AAT GCA GAT GGA CGT TTT GAA GGG ACC AGT ACG AAA ACA Leu Phe Asp Asn Ala Asp Gly Arg Phe Glu Gly Thr Ser Thr Lys Thr 135 140 145 150	604
GTC ATC AGG TAC AAT GGC ACT GTC ACC TGG ACT CCA CCG GCA AAC TAC Val Ile Arg Tyr Asn Gly Thr Val Thr Trp Thr Pro Pro Ala Asn Tyr 155 160 165	652
AAA AGT TCC TGT ACC ATA GAT GTC ACG TTT TTC CCA TTT GAC CTT CAG Lys Ser Ser Cys Thr Ile Asp Val Thr Phe Phe Pro Phe Asp Leu Gln 170 175 180	700
AAC TGT TCC ATG AAA TTT GGT TCT TGG ACT TAT GAT GGA TCA CAG GTT Asn Cys Ser Met Lys Phe Gly Ser Trp Thr Tyr Asp Gly Ser Gln Val 185 190 195	748
GAT ATA ATT CTA GAG GAC CAA GAT GTA GAC AAG AGA GAT TTT TTT GAT Asp Ile Ile Leu Glu Asp Gln Asp Val Asp Lys Arg Asp Phe Phe Asp 200 205 210	796
AAT GGA GAA TGG GAG ATT GTG AGT GCA ACA GGG AGC AAA GGA AAC AGA Asn Gly Glu Trp Glu Ile Val Ser Ala Thr Gly Ser Lys Gly Asn Arg 215 220 225 230	844
ACC GAC AGC TGT TGC TGG TAT CCG TAT GTC ACT TAC TCA TTT GTA ATC Thr Asp Ser Cys Cys Trp Tyr Pro Tyr Val Thr Tyr Ser Phe Val Ile 235 240 245	892
AAG CGC CTG CCT CTC TTT TAT ACC TTG TTC CTT ATA ATA CCC TGT ATT Lys Arg Leu Pro Leu Phe Tyr Thr Thr Phe Leu Ile Ile Pro Cys Ile 250 255 260	940
GGG CTC TCA TTT TTA ACT GTA CTT GTC TTC TAT CTT CCT TCA AAT GAA Gly Leu Ser Phe Leu Thr Val Leu Val Phe Tyr Leu Pro Ser Asn Glu 265 270 275	988



GGT GAA AAG ATT TGT CTC TGC ACT TCA GTA CTT GTG TCT TTG ACT GTC Gly Glu Lys Ile Cys Leu Cys Thr Ser Val Leu Val Ser Leu Thr Val 280 285 290	1036
TTC CTT CTG GTT AAT GAA GAG ATC ATA CCA TCA TCT TCA AAA GTC ATA Phe Leu Leu Val Ile Glu Glu Ile Ile Pro Ser Ser Ser Lys Val Ile 295 300 305 310	1084
CCT CTA ATT GGA GAG TAT CTG GTA TTT ACC ATG ATT TTT GTG ACA CTG Pro Leu Ile Gly Glu Tyr Leu Val Phe Thr Met Ile Phe Val Thr Leu 315 320 325	1132
TCA ATT ATG GTA ACC GTC TTC GCT ATC AAC ATT CAT CAT CGT TCT TCC Ser Ile Met Val Thr Val Phe Ala Ile Asn Ile His His Arg Ser Ser 330 335 340	1180
TCA ACA CAT AAT GCC ATG GCG CCT TTG GTC CGC AAG ATA TTT CTT CAC Ser Thr His Asn Ala Met Ala Pro Leu Val Arg Lys Ile Phe Leu His 345 350 355	1228
ACG CTT CCC AAA CTG CTT TGC ATG AGA AGT CAT GTA GAC AGG TAC TTC Thr Leu Pro Lys Leu Leu Cys Met Arg Ser His Val Asp Arg Tyr Phe 360 365 370	1276
ACT CAG AAA GAG GAA ACT GAG AGT GGT AGT GGA CCA AAA TCT TCT AGA Thr Gln Lys Glu Glu Thr Glu Ser Gly Ser Gly Pro Lys Ser Ser Arg 375 380 385 390	1324
AAC ACA TTG GAA GCT GCG CTC AAT TCT ATT CGC TAC ATT ACA AGA CAC Asn Thr Leu Glu Ala Ala Leu Asn Ser Ile Arg Tyr Ile Thr Arg His 395 400 405	1372
ATC ATG AAG GAA AAT GAT GTC CGT GAG GTT GTT GAA GAT TGG AAA TTC Ile Met Lys Glu Asn Asp Val Arg Glu Val Val Glu Asp Trp Lys Phe 410 415 420	1420
ATA GCC CAG GTT CTT GAT CGG ATG TTT CTG TGG ACT TTT CTT TTC GTT Ile Ala Gln Val Leu Asp Arg Met Phe Leu Trp Thr Phe Leu Phe Val 425 430 435	1468
TCA ATT GTT GGA TCT CTT GGG CTT TTT GTT CCT GTT ATT TAT AAA TGG Ser Ile Val Gly Ser Leu Gly Leu Phe Val Pro Val Ile Tyr Lys Trp 440 445 450	1516
GCA AAT ATA TTA ATA CCA GTT CAT ATT GGA AAT GCA AAT AAG TGAAGCCTCC Ala Asn Ile Leu Ile Pro Val His Ile Gly Asn Ala Asn Lys 455 460 465	1568
CAAGGGACTG AAGTATACAT TTAGTTAACA CACATATATC TGATGGCACC TATAAAATTA	1628
TGAAAATGTA AGTTATGTGT TAAATTTAGT GCAAGCTTTA ACAGACTAAG TTGCTAACCT	1688
CAATTTTATGT TAACAGATGA TCCATTTGAA CAGTTGGCTG TATGACTGAA GTAATAACTG	1748
ATGAGATACA TTTGATCTTG TAAAAATAGC AAAATATTAT CTGAACTGGA CTAGTGAAAA	1808
ATCTAGTATT TGTATCCTGG	1828

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 468 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Ala Ala Arg Gly Ser Gly Pro Arg Ala Leu Arg Leu Leu Leu Leu  
1 5 10 15  
Val Gln Leu Val Ala Gly Arg Cys Gly Leu Ala Gly Ala Ala Gly Gly  
20 25 30  
Ala Gln Arg Gly Leu Ser Glu Pro Ser Ser Ile Ala Lys His Glu Asp  
35 40 45  
Ser Leu Leu Lys Asp Leu Phe Gln Asp Tyr Glu Arg Trp Val Arg Pro  
50 55 60  
Val Glu His Leu Asn Asp Lys Ile Lys Ile Lys Phe Gly Leu Ala Ile  
65 70 75 80  
Ser Gln Leu Val Asp Val Asp Glu Lys Asn Gln Leu Met Thr Thr Asn  
85 90 95  
Val Trp Leu Lys Gln Glu Trp Ile Asp Val Lys Leu Arg Trp Asn Pro  
100 105 110  
Asp Asp Tyr Gly Gly Ile Lys Val Ile Arg Val Pro Ser Asp Ser Val  
115 120 125  
Trp Thr Pro Asp Ile Val Leu Phe Asp Asn Ala Asp Gly Arg Phe Glu  
130 135 140  
Gly Thr Ser Thr Lys Thr Val Ile Arg Tyr Asn Gly Thr Val Thr Trp  
145 150 155 160  
Thr Pro Pro Ala Asn Tyr Lys Ser Ser Cys Thr Ile Asp Val Thr Phe  
165 170 175  
Phe Pro Phe Asp Leu Gln Asn Cys Ser Met Lys Phe Gly Ser Trp Thr  
180 185 190  
Tyr Asp Gly Ser Gln Val Asp Ile Ile Leu Glu Asp Gln Asp Val Asp  
195 200 205  
Lys Arg Asp Phe Phe Asp Asn Gly Glu Trp Glu Ile Val Ser Ala Thr  
210 215 220  
Gly Ser Lys Gly Asn Arg Thr Asp Ser Cys Cys Trp Tyr Pro Tyr Val  
225 230 235 240  
Thr Tyr Ser Phe Val Ile Lys Arg Leu Pro Leu Phe Tyr Thr Leu Phe  
245 250 255  
Leu Ile Ile Pro Cys Ile Gly Leu Ser Phe Leu Thr Val Leu Val Phe  
260 265 270  
Tyr Leu Pro Ser Asn Glu Gly Glu Lys Ile Cys Leu Cys Thr Ser Val  
275 280 285  
Leu Val Ser Leu Thr Val Phe Leu Leu Val Ile Glu Glu Ile Ile Pro  
290 295 300  
Ser Ser Ser Lys Val Ile Pro Leu Ile Gly Glu Tyr Leu Val Phe Thr  
305 310 315 320  
Met Ile Phe Val Thr Leu Ser Ile Met Val Thr Val Phe Ala Ile Asn  
325 330 335  
Ile His His Arg Ser Ser Ser Thr His Asn Ala Met Ala Pro Leu Val  
340 345 350

Arg Lys Ile Phe Leu His Thr Leu Pro Lys Leu Leu Cys Met Arg Ser  
 355 360 365  
 His Val Asp Arg Tyr Phe Thr Gln Lys Glu Glu Thr Glu Ser Gly Ser  
 370 375 380  
 Gly Pro Lys Ser Ser Arg Asn Thr Leu Glu Ala Ala Leu Asn Ser Ile  
 385 390 395 400  
 Arg Tyr Ile Thr Arg His Ile Met Lys Glu Asn Asp Val Arg Glu Val  
 405 410 415  
 Val Glu Asp Trp Lys Phe Ile Ala Gln Val Leu Asp Arg Met Phe Leu  
 420 425 430  
 Trp Thr Phe Leu Phe Val Ser Ile Val Gly Ser Leu Gly Leu Phe Val  
 435 440 445  
 Pro Val Ile Tyr Lys Trp Ala Asn Ile Leu Ile Pro Val His Ile Gly  
 450 455 460  
 Asn Ala Asn Lys  
 465

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1743 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 143..1627
- (D) OTHER INFORMATION: /product= "ALPHA-6 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGGTTTTGA TTTCTGAGAA GACACACACG GATTGCAGTG GGCTTCTGAT GATGTCAAGG	60
TTGGATGCAT GTGGCTGACT GATAGCTCTT TGTTCCTCAC AATCCTTTGC CTAGGAAAAA	120
GGAATCCAAG TGTGTTTTAA CC ATG CTG ACC AGC AAG GGG CAG GGA TTC CTT	172
Met Leu Thr Ser Lys Gly Gln Gly Phe Leu	
1 5 10	
CAT GGG GGC TTG TGT CTC TGG CTG TGT GTG TTC ACA CCT TTC TTT AAA	220
His Gly Gly Leu Cys Leu Trp Leu Cys Val Phe Thr Pro Phe Phe Lys	
15 20 25	
GGC TGT GTG GGC TGT GCA ACT GAG GAG AGG CTC TTC CAC AAA CTG TTT	268
Gly Cys Val Gly Cys Ala Thr Glu Glu Arg Leu Phe His Lys Leu Phe	
30 35 40	
TCT CAT TAC AAC CAG TTC ATC AGG CCT GTG GAA AAC GTT TCC GAC CCT	316
Ser His Tyr Asn Gln Phe Ile Arg Pro Val Glu Asn Val Ser Asp Pro	
45 50 55	
GTC ACC GTA CAC TTT GAA GTG GCC ATC ACC CAG CTG GCC AAC GTG GAT	364
Val Thr Val His Phe Glu Val Ala Ile Thr Gln Leu Ala Asn Val Asp	
60 65 70	

GAA GTA AAC CAG ATC ATG GAA ACC AAT TTG TGG CTG CGT CAC ATC TGG Glu Val Asn Gln Ile Met Glu Thr Asn Leu Trp Leu Arg His Ile Trp 75 80 85 90	412
AAT GAT TAT AAA TTG CGC TGG GAT CCA ATG GAA TAT GAT GGC ATT GAG Asn Asp Tyr Lys Leu Arg Trp Asp Pro Met Glu Tyr Asp Gly Ile Glu 95 100 105	460
ACT CTT CGC GTT CCT GCA GAT AAG ATT TGG AAG CCC GAC ATT GTT CTC Thr Leu Arg Val Pro Ala Asp Lys Ile Trp Lys Pro Asp Ile Val Leu 110 115 120	508
TAT AAC AAT GCT GTT GGT GAC TTC CAA GTA GAA GGC AAA ACA AAA GCT Tyr Asn Asn Ala Val Gly Asp Phe Gln Val Glu Gly Lys Thr Lys Ala 125 130 135	556
CTT CTT AAA TAC AAT GGC ATG ATA ACC TGG ACT CCA CCA GCT ATT TTT Leu Leu Lys Tyr Asn Gly Met Ile Thr Trp Thr Pro Pro Ala Ile Phe 140 145 150	604
AAG AGT TCC TGC CCT ATG GAT ATC ACC TTT TTC CCT TTT GAT CAT CAA Lys Ser Ser Cys Pro Met Asp Ile Thr Phe Phe Pro Phe Asp His Gln 155 160 165 170	652
AAC TGT TCC CTA AAA TTT GGT TCC TGG ACG TAT GAC AAA GCT GAA ATT Asn Cys Ser Leu Lys Phe Gly Ser Trp Thr Tyr Asp Lys Ala Glu Ile 175 180 185	700
GAT CTT CTA ATC ATT GGA TCA AAA GTG GAT ATG AAT GAT TTT TGG GAA Asp Leu Leu Ile Ile Gly Ser Lys Val Asp Met Asn Asp Phe Trp Glu 190 195 200	748
AAC AGT GAA TGG GAA ATC ATT GAT GCC TCT GGC TAC AAA CAT GAC ATC Asn Ser Glu Trp Glu Ile Ile Asp Ala Ser Gly Tyr Lys His Asp Ile 205 210 215	796
AAA TAC AAC TGT TGT GAA GAG ATA TAC ACA GAT ATA ACC TAT TCT TTC Lys Tyr Asn Cys Cys Glu Glu Ile Tyr Thr Asp Ile Thr Tyr Ser Phe 220 225 230	844
TAC ATT AGA AGA TTG CCG ATG TTT TAC ACG ATT AAT CTG ATC ATC CCT Tyr Ile Arg Arg Leu Pro Met Phe Tyr Thr Ile Asn Leu Ile Ile Pro 235 240 245 250	892
TGT CTC TTT ATT TCA TTT CTA ACC GTG TTG GTC TTT TAC CTT CCT TCG Cys Leu Phe Ile Ser Phe Leu Thr Val Leu Val Phe Tyr Leu Pro Ser 255 260 265	940
GAC TGT GGT GAA AAA GTG ACG CTT TGT ATT TCA GTC CTG CTT TCT CTG Asp Cys Gly Glu Lys Val Thr Leu Cys Ile Ser Val Leu Leu Ser Leu 270 275 280	988
ACT GTG TTT TTG CTG GTC ATC ACA GAA ACC ATC CCA TCC ACA TCT CTG Thr Val Phe Leu Leu Val Ile Thr Glu Thr Ile Pro Ser Thr Ser Leu 285 290 295	1036
GTG GTC CCA CTG GTG GGT GAG TAC CTG CTG TTC ACC ATG ATC TTT GTC Val Val Pro Leu Val Gly Glu Tyr Leu Leu Phe Thr Met Ile Phe Val 300 305 310	1084
ACA CTG TCC ATC GTG GTG ACT GTG TTT GTG TTG AAC ATA CAC TAC CGC Thr Leu Ser Ile Val Val Thr Val Phe Val Leu Asn Ile His Tyr Arg 315 320 325 330	1132
ACC CCA ACC ACG CAC ACA ATG CCC AGG TGG GTG AAG ACA GTT TTC CTG Thr Pro Thr Thr His Thr Met Pro Arg Trp Val Lys Thr Val Phe Leu 335 340 345	1180

AAG CTG CTG CCC CAG GTC CTG CTG ATG AGG TGG CCT CTG GAC AAG ACA Lys Leu Leu Pro Gln Val Leu Leu Met Arg Trp Pro Leu Asp Lys Thr 350 355 360	1228
AGG GGC ACA GGC TCT GAT GCA GTG CCC AGA GGC CTT GCC AGG AGG CCT Arg Gly Thr Gly Ser Asp Ala Val Pro Arg Gly Leu Ala Arg Arg Pro 365 370 375	1276
GCC AAA GGC AAG CTT GCA AGC CAT GGG GAA CCC AGA CAT CTT AAA GAA Ala Lys Gly Lys Leu Ala Ser His Gly Glu Pro Arg His Leu Lys Glu 380 385 390	1324
TGC TTC CAT TGT CAC AAA TCA AAT GAG CTT GCC ACA AGC AAG AGA AGA Cys Phe His Cys His Lys Ser Asn Glu Leu Ala Thr Ser Lys Arg Arg 395 400 405 410	1372
TTA AGT CAT CAG CCA TTA CAG TGG GTG GTG GAA AAT TCG GAG CAC TCG Leu Ser His Gln Pro Leu Gln Trp Val Val Glu Asn Ser Glu His Ser 415 420 425	1420
CCT GAA GTT GAA GAT GTG ATT AAC AGT GTT CAG TTC ATA GCA GAA AAC Pro Glu Val Glu Asp Val Ile Asn Ser Val Gln Phe Ile Ala Glu Asn 430 435 440	1468
ATG AAG AGC CAC AAT GAA ACC AAG GAG GTA GAA GAT GAC TGG AAA TAC Met Lys Ser His Asn Glu Thr Lys Glu Val Glu Asp Asp Trp Lys Tyr 445 450 455	1516
GTG GCC ATG GTG GTG GAC AGA GTA TTT CTT TGG GTA TTT ATA ATT GTC Val Ala Met Val Val Asp Arg Val Phe Leu Trp Val Phe Ile Ile Val 460 465 470	1564
TGT GTA TTT GGA ACT GCA GGG CTA TTT CTA CAG CCA CTA CTT GGG AAC Cys Val Phe Gly Thr Ala Gly Leu Phe Leu Gln Pro Leu Leu Gly Asn 475 480 485 490	1612
ACA GGA AAA TCT TAAATGTAT TTTCTTTTAT GTTCAGAAAT TTACAGACAC Thr Gly Lys Ser 495	1664
CATATTTGTT CTGCATTCCC TGCCACAAGG AAAGGAAAGC AAAGGCTTCC CACCCAAGTC	1724
CCCCATCTGC TAAAACCCG	1743

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 494 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Leu Thr Ser Lys Gly Gln Gly Phe Leu His Gly Gly Leu Cys Leu 1 5 10 15
Trp Leu Cys Val Phe Thr Pro Phe Phe Lys Gly Cys Val Gly Cys Ala 20 25 30
Thr Glu Glu Arg Leu Phe His Lys Leu Phe Ser His Tyr Asn Gln Phe 35 40 45
Ile Arg Pro Val Glu Asn Val Ser Asp Pro Val Thr Val His Phe Glu 50 55 60

Val	Ala	Ile	Thr	Gln	Leu	Ala	Asn	Val	Asp	Glu	Val	Asn	Gln	Ile	Met	65	70	75	80
Glu	Thr	Asn	Leu	Trp	Leu	Arg	His	Ile	Trp	Asn	Asp	Tyr	Lys	Leu	Arg	85	90	95	
Trp	Asp	Pro	Met	Glu	Tyr	Asp	Gly	Ile	Glu	Thr	Leu	Arg	Val	Pro	Ala	100	105	110	
Asp	Lys	Ile	Trp	Lys	Pro	Asp	Ile	Val	Leu	Tyr	Asn	Asn	Ala	Val	Gly	115	120	125	
Asp	Phe	Gln	Val	Glu	Gly	Lys	Thr	Lys	Ala	Leu	Leu	Lys	Tyr	Asn	Gly	130	135	140	
Met	Ile	Thr	Trp	Thr	Pro	Pro	Ala	Ile	Phe	Lys	Ser	Ser	Cys	Pro	Met	145	150	155	160
Asp	Ile	Thr	Phe	Phe	Pro	Phe	Asp	His	Gln	Asn	Cys	Ser	Leu	Lys	Phe	165	170	175	
Gly	Ser	Trp	Thr	Tyr	Asp	Lys	Ala	Glu	Ile	Asp	Leu	Leu	Ile	Ile	Gly	180	185	190	
Ser	Lys	Val	Asp	Met	Asn	Asp	Phe	Trp	Glu	Asn	Ser	Glu	Trp	Glu	Ile	195	200	205	
Ile	Asp	Ala	Ser	Gly	Tyr	Lys	His	Asp	Ile	Lys	Tyr	Asn	Cys	Cys	Glu	210	215	220	
Glu	Ile	Tyr	Thr	Asp	Ile	Thr	Tyr	Ser	Phe	Tyr	Ile	Arg	Arg	Leu	Pro	225	230	235	240
Met	Phe	Tyr	Thr	Ile	Asn	Leu	Ile	Ile	Pro	Cys	Leu	Phe	Ile	Ser	Phe	245	250	255	
Leu	Thr	Val	Leu	Val	Phe	Tyr	Leu	Pro	Ser	Asp	Cys	Gly	Glu	Lys	Val	260	265	270	
Thr	Leu	Cys	Ile	Ser	Val	Leu	Leu	Ser	Leu	Thr	Val	Phe	Leu	Leu	Val	275	280	285	
Ile	Thr	Glu	Thr	Ile	Pro	Ser	Thr	Ser	Leu	Val	Val	Pro	Leu	Val	Gly	290	295	300	
Glu	Tyr	Leu	Leu	Phe	Thr	Met	Ile	Phe	Val	Thr	Leu	Ser	Ile	Val	Val	305	310	315	320
Thr	Val	Phe	Val	Leu	Asn	Ile	His	Tyr	Arg	Thr	Pro	Thr	Thr	His	Thr	325	330	335	
Met	Pro	Arg	Trp	Val	Lys	Thr	Val	Phe	Leu	Lys	Leu	Leu	Pro	Gln	Val	340	345	350	
Leu	Leu	Met	Arg	Trp	Pro	Leu	Asp	Lys	Thr	Arg	Gly	Thr	Gly	Ser	Asp	355	360	365	
Ala	Val	Pro	Arg	Gly	Leu	Ala	Arg	Arg	Pro	Ala	Lys	Gly	Lys	Leu	Ala	370	375	380	
Ser	His	Gly	Glu	Pro	Arg	His	Leu	Lys	Glu	Cys	Phe	His	Cys	His	Lys	385	390	395	400
Ser	Asn	Glu	Leu	Ala	Thr	Ser	Lys	Arg	Arg	Leu	Ser	His	Gln	Pro	Leu	405	410	415	

Gln Trp Val Val Glu Asn Ser Glu His Ser Pro Glu Val Glu Asp Val  
420 425 430  
Ile Asn Ser Val Gln Phe Ile Ala Glu Asn Met Lys Ser His Asn Glu  
435 440 445  
Thr Lys Glu Val Glu Asp Asp Trp Lys Tyr Val Ala Met Val Val Asp  
450 455 460  
Arg Val Phe Leu Trp Val Phe Ile Ile Val Cys Val Phe Gly Thr Ala  
465 470 475 480  
Gly Leu Phe Leu Gln Pro Leu Leu Gly Asn Thr Gly Lys Ser  
485 490

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1876 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 73..1581  
(D) OTHER INFORMATION: /product= "ALPHA-7 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCCGCAGGC GCAGGCCCGG GGCACAGCCG AGACGTGGAG CGCGCCGGCT CGCTGCAGCT	60
CCGGGACTCA ACATGCGCTG CTCGCCGGA GCGCTCTGGC TGGCGCTGGC CGCGTCGCTC	120
CTGCACGTGT CCCTGCAAGG CGAGTTCAG AGGAAGCTTT ACAAGGAGCT GGTCAAGAAC	180
TACAATCCCT TGGAGAGGCC CGTGGCCAAT GACTCGCAAC CACTCACCCT CTACTTCTCC	240
CTGAGCCTCC TGCAGATCAT GGACGTGGAT GAGAAGAACC AAGTTTTAAC CACCAACATT	300
TGGCTGCAAA TGTCTTGAC AGATCACTAT TTACAGTGA ATGTGTCAGA ATATCCAGGG	360
GTGAAGACTG TTCGTTTCCC AGATGGCCAG ATTTGGAAC CAGACATTCT TCTCTATAAC	420
AGTGCTGATG AGCGCTTGA CGCCACATTC CACACTAACG TGTGGTGAA TTCTTCTGGG	480
CATTGCCAGT ACCTGCCTCC AGGCATATTC AAGAGTTCCT GCTACATCGA TGTAAGCTGG	540
TTTCCCTTTG ATGTGCAGCA CTGCAAACTG AAGTTTGGGT CCTGGTCTTA CGGAGGCTGG	600
TCCTTGATC TGCAGATGCA GGAGGCAGAT ATCAGTGGCT ATATCCCCAA TGGAGAATGG	660
GACCTAGTGG GAATCCCCCG CAAGAGGAGT GAAAGGTTCT ATGAGTGCTG CAAAGAGCCC	720
TACCCCGATG TCACCTTCAC AGTGACCATG CGCCGCAGGA CGCTCTACTA TGGCCTCAAC	780
CTGCTGATCC CCTGTGTGCT CATCTCCGCC CTCGCCCTGC TGGTGTTCCT GCTTCTGCA	840
GATTCCGGGG AGAAGATTTT CCTGGGGATA AAGTCTTAC TCTCTCTTAC CGTCTTCATG	900
CTGCTCGTGG CTGAGATCAT GCGCGCAACA TCCGATTCGG TACCATTGAT AGCCCAATAC	960
TTGCCAGCA CCATGATCAT CGTGGGCTTC TCGGTGGTGG TGACGGTGAT CGTCTGCAG	1020

TACCACCACC ACGACCCCGA CCGGGGCAAG ATGCCCAAGT GGACCAGAGT CATCCTTCTG 1080  
AACTGGTGCG CGTGGTTTCT SCGAATGAAG AGGCCCCGGG AGGACAAGGT GCGCCCCGGC 1140  
TGCCAGCACA AGCAGCGGCG CTGCAGCCTG GCCAGTGTGG AGATGAGCGC CGTGGCGCGG 1200  
CCGCCCCCCA GCAACGGGAA CCTGCTGTAC ATCGGCTTCC GCGGCTTGA CCGCGTGCAC 1260  
TGTGTCCCGA CCCCCGACTC TGGGGTAGTG TGTGGCCGCA TGGCCTGCTC CCCCACGCAC 1320  
GATGAGCACC TCCTGCACGG CCGGCAACCC CCCGAGGGGG ACCCGGACTT GGCCAAGATC 1380  
CTGGAGGAGG TCCGCTACAT TGCCAATCGC TTCGCTGCC AGGACGAAAG CGAGGCGGTC 1440  
TGCAGCGAGT GGAAGTTGCG CGCTGTGTG GTGGACCGCC TGTGCCTCAT GGCTTCTCG 1500  
GTCTTCACCA TCATCTGCAC CATCGGCATC CTGATGTGG CTCCCAACTT CGTGGAGGCC 1560  
GTGTCCAAAG ACTTTGCGTA ACCACGCCTG GTTCTGTACA TGTGGAAAAC TCACAGATGG 1620  
GCAAGGCCTT TGGCTTGGCG AGATTGGGG GTGCTAATCC AGGACAGCAT TACACGCCAC 1680  
AACTCCAGTG TTCCTTCTG GCTGTGAGTC GTGTTGCTTA CCGTTTCTTT GTTACTTTAG 1740  
GTAGTAGAAT CTCAGCACTT TGTTCATAT TCTCAGATGG GCTGATAGAT ATCCTTGGCA 1800  
CATCCGTACC ATCGGTCAGC AGGGCCACTG AATAGTCATT TTGCCCATTA GCCCCTGCC 1860  
TGGAAGCCCC TTCGGA 1876

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 502 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Arg Cys Ser Pro Gly Gly Val Trp Leu Ala Leu Ala Ala Ser Leu  
1 5 10 15  
Leu His Val Ser Leu Gln Gly Glu Phe Gln Arg Lys Leu Tyr Lys Glu  
20 25 30  
Leu Val Lys Asn Tyr Asn Pro Leu Glu Arg Pro Val Ala Asn Asp Ser  
35 40 45  
Gln Pro Leu Thr Val Tyr Phe Ser Leu Ser Leu Leu Gln Ile Met Asp  
50 55 60  
Val Asp Glu Lys Asn Gln Val Leu Thr Thr Asn Ile Trp Leu Gln Met  
65 70 75 80  
Ser Trp Thr Asp His Tyr Leu Gln Trp Asn Val Ser Glu Tyr Pro Gly  
85 90 95  
Val Lys Thr Val Arg Phe Pro Asp Gly Gln Ile Trp Lys Pro Asp Ile  
100 105 110  
Leu Leu Tyr Asn Ser Ala Asp Glu Arg Phe Asp Ala Thr Phe His Thr  
115 120 125  
Asn Val Leu Val Asn Ser Ser Gly His Cys Gln Tyr Leu Pro Pro Gly  
130 135 140



Ile Phe Lys Ser Ser Cys Tyr Ile Asp Val Arg Trp Phe Pro Phe Asp  
 145 150 155 160  
 Val Gln His Cys Lys Leu Lys Phe Gly Ser Trp Ser Tyr Gly Gly Trp  
 165 170 175  
 Ser Leu Asp Leu Gln Met Gln Glu Ala Asp Ile Ser Gly Tyr Ile Pro  
 180 185 190  
 Asn Gly Glu Trp Asp Leu Val Gly Ile Pro Gly Lys Arg Ser Glu Arg  
 195 200 205  
 Phe Tyr Glu Cys Cys Lys Glu Pro Tyr Pro Asp Val Thr Phe Thr Val  
 210 215 220  
 Thr Met Arg Arg Arg Thr Leu Tyr Tyr Gly Leu Asn Leu Leu Ile Pro  
 225 230 235 240  
 Cys Val Leu Ile Ser Ala Leu Ala Leu Leu Val Phe Leu Leu Pro Ala  
 245 250 255  
 Asp Ser Gly Glu Lys Ile Ser Leu Gly Ile Thr Val Leu Leu Ser Leu  
 260 265 270  
 Thr Val Phe Met Leu Leu Val Ala Glu Ile Met Pro Ala Thr Ser Asp  
 275 280 285  
 Ser Val Pro Leu Ile Ala Gln Tyr Phe Ala Ser Thr Met Ile Ile Val  
 290 295 300  
 Gly Leu Ser Val Val Val Thr Val Ile Val Leu Gln Tyr His His His  
 305 310 315 320  
 Asp Pro Asp Gly Gly Lys Met Pro Lys Trp Thr Arg Val Ile Leu Leu  
 325 330 335  
 Asn Trp Cys Ala Trp Phe Leu Arg Met Lys Arg Pro Gly Glu Asp Lys  
 340 345 350  
 Val Arg Pro Ala Cys Gln His Lys Gln Arg Arg Cys Ser Leu Ala Ser  
 355 360 365  
 Val Glu Met Ser Ala Val Ala Pro Pro Pro Ala Ser Asn Gly Asn Leu  
 370 375 380  
 Leu Tyr Ile Gly Phe Arg Gly Leu Asp Gly Val His Cys Val Pro Thr  
 385 390 395 400  
 Pro Asp Ser Gly Val Val Cys Gly Arg Met Ala Cys Ser Pro Thr His  
 405 410 415  
 Asp Glu His Leu Leu His Gly Gly Gln Pro Pro Glu Gly Asp Pro Asp  
 420 425 430  
 Leu Ala Lys Ile Leu Glu Glu Val Arg Tyr Ile Ala Asn Arg Phe Arg  
 435 440 445  
 Cys Gln Asp Glu Ser Glu Ala Val Cys Ser Glu Trp Lys Phe Ala Ala  
 450 455 460  
 Cys Val Val Asp Arg Leu Cys Leu Met Ala Phe Ser Val Phe Thr Ile  
 465 470 475 480  
 Ile Cys Thr Ile Gly Ile Leu Met Ser Ala Pro Asn Phe Val Glu Ala  
 485 490 495

Val Ser Lys Asp Phe Ala  
500

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2448 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 265..1773
- (D) OTHER INFORMATION: /product= "BETA-2 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCCTCCCCC TCACCGTCCC AATTGTATTC CCTGGAAGAG CAGCCGGAAA AGCCTCCGCC	60
TGCTCATACC AGGATAGGCA AGAAGCTGGT TTCTCCTCGC AGCCGGCTCC CTGAGGCCCA	120
GGAACCACCG CGCGGCCCGG CACCACCTGG ACCCAGCTCC AGGCGGGCGC GGCTTCAGCA	180
CCACGGACAG CGCCCCACCC GCGGCCCTCC CCCC GGCGGC GCGCTCCAGC CGGTGTAGGC	240
GAGGCAGCGA GCTATGCCCC CGGC ATG GCC CGC TGC GGC CCC GTG GCG	291
Met Ala Arg Arg Cys Gly Pro Val Ala	
1 5	
CTG CTC CTT GGC TTC GGC CTC CTC CGG CTG TGC TCA GGG GTG TGG GGT	339
Leu Leu Leu Gly Phe Gly Leu Leu Arg Leu Cys Ser Gly Val Trp Gly	
10 15 20 25	
ACG GAT ACA GAG GAG CGG CTG GTG GAG CAT CTC CTG GAT CCT TCC CGC	387
Thr Asp Thr Glu Glu Arg Leu Val Glu His Leu Leu Asp Pro Ser Arg	
30 35 40	
TAC AAC AAG CTT ATC CGC CCA GCC ACC AAT GGC TCT GAG CTG GTG ACA	435
Tyr Asn Lys Leu Ile Arg Pro Ala Thr Asn Gly Ser Glu Leu Val Thr	
45 50 55	
GTA CAG CTT ATG GTG TCA CTG GCC CAG CTC ATC AGT GTG CAT GAG CGG	483
Val Gln Leu Met Val Ser Leu Ala Gln Leu Ile Ser Val His Glu Arg	
60 65 70	
GAG CAG ATC ATG ACC ACC AAT GTC TGG CTG ACC CAG GAG TGG GAA GAT	531
Glu Gln Ile Met Thr Thr Asn Val Trp Leu Thr Gln Glu Trp Glu Asp	
75 80 85	
TAT CGC CTC ACC TGG AAG CCT GAA GAG TTT GAC AAC ATG AAG AAA GTT	579
Tyr Arg Leu Thr Trp Lys Pro Glu Glu Phe Asp Asn Met Lys Lys Val	
90 95 100 105	
CGG CTC CCT TCC AAA CAC ATC TGG CTC CCA GAT GTG GTC CTG TAC AAC	627
Arg Leu Pro Ser Lys His Ile Trp Leu Pro Asp Val Val Leu Tyr Asn	
110 115 120	
AAT GCT GAC GGC ATG TAC GAG GTG TCC TTC TAT TCC AAT GCC GTG GTC	675
Asn Ala Asp Gly Met Tyr Glu Val Ser Phe Tyr Ser Asn Ala Val Val	
125 130 135	
TCC TAT GAT GGC AGC ATC TTC TGG CTG CCG CCT GCC ATC TAC AAG AGC	723
Ser Tyr Asp Gly Ser Ile Phe Trp Leu Pro Pro Ala Ile Tyr Lys Ser	
140 145 150	

GCA TGC AAG ATT GAA GTA AAG CAC TTC CCA TTT GAC CAG CAG AAC TGC Ala Cys Lys Ile Glu Val Lys His Phe Pro Phe Asp Gln Gln Asn Cys 155 160 165	771
ACC ATG AAG TTC CGT TCG TGG ACC TAC GAC CGC ACA GAG ATC GAC TTG Thr Met Lys Phe Arg Ser Trp Thr Tyr Asp Arg Thr Glu Ile Asp Leu 170 175 180 185	819
GTG CTG AAG AGT GAG GTG GCC AGC CTG GAC GAC TTC ACA CCT AGT GGT Val Leu Lys Ser Glu Val Ala Ser Leu Asp Asp Phe Thr Pro Ser Gly 190 195 200	867
GAG TGG GAC ATC GTG GCG CTG CCG GGC CGG CGC AAC GAG AAC CCC GAC Glu Trp Thr Asp Ile Val Ala Leu Pro Gly Arg Arg Asn Glu Asn Pro Asp 205 210 215	915
GAC TCT ACG TAC GTG GAC ATC ACG TAT GAC TTC ATC ATT CGC CGC AAG Asp Ser Thr Tyr Val Asp Ile Thr Tyr Asp Phe Ile Ile Arg Arg Lys 220 225 230	963
CCG CTC TTC TAC ACC ATC AAC CTC ATC ATC CCC TGT GTG CTC ATC ACC Pro Leu Phe Tyr Thr Ile Asn Leu Ile Ile Pro Cys Val Leu Ile Thr 235 240 245	1011
TCG CTA GCC ATC CTT GTC TTC TAC CTG CCA TCC GAC TGT GGC GAG AAG Ser Leu Ala Ile Leu Val Phe Tyr Leu Pro Ser Asp Cys Gly Glu Lys 250 255 260 265	1059
ATG ACG TTG TGC ATC TCA GTG CTG CTG GCG CTC ACC GTC TTC CTG CTG Met Thr Leu Cys Ile Ser Val Leu Leu Ala Leu Thr Val Phe Leu Leu 270 275 280	1107
CTC ATC TCC AAG ATC GTG CCT CCC ACC TCC CTC GAC GTG CCG CTC GTC Leu Ile Ser Lys Ile Val Pro Pro Thr Ser Leu Asp Val Pro Leu Val 285 290 295	1155
GGC AAG TAC CTC ATG TTC ACC ATG GTG CTT GTC ACC TTC TCC ATC GTC Gly Lys Tyr Leu Met Phe Thr Met Val Leu Val Thr Phe Ser Ile Val 300 305 310	1203
ACC AGC GTG TGC GTG CTC AAC GTG CAC CAC CGC TCG CCC ACC ACG CAC Thr Ser Val Cys Val Leu Asn Val His His Arg Ser Pro Thr Thr His 315 320 325	1251
ACC ATG GCG CCC TGG GTG AAG GTC GTC TTC CTG GAG AAG CTG CCC GCG Thr Met Ala Pro Trp Val Lys Val Val Phe Leu Glu Lys Leu Pro Ala 330 335 340 345	1299
CTG CTC TTC ATG CAG CAG CCA CGC CAT CAT TGC GCC CGT CAG CGC CTG Leu Leu Phe Met Gln Gln Pro Arg His His Cys Ala Arg Gln Arg Leu 350 355 360	1347
CGC CTG CCG CGA CGC CAG CGT GAG CGC GAG GGC GCT GGA GCC CTC TTC Arg Leu Arg Arg Arg Gln Arg Glu Arg Glu Gly Ala Gly Ala Leu Phe 365 370 375	1395
TTC CGC GAA GCC CCA GGG GCC GAC TCC TGC ACG TGC TTC GTC AAC CGC Phe Arg Glu Ala Pro Gly Ala Asp Ser Cys Thr Cys Phe Val Asn Arg 380 385 390	1443
GCG TCG GTG CAG GCG TTG GCC GGG GCC TTC GGG GCT GAG CCT GCA CCA Ala Ser Val Gln Gly Leu Ala Gly Ala Phe Gly Ala Glu Pro Ala Pro 395 400 405	1491
GTG GCG GGC CCC GGG CGC TCA GGG GAG CCG TGT GGC TGT GGC CTC CGG Val Ala Gly Pro Gly Arg Ser Gly Glu Pro Cys Gly Cys Gly Leu Arg 410 415 420 425	1539

GAG GCG GTG GAC GGC GTG CGC TTC ATC GCA GAC CAC ATG CGG AGC GAG Glu Ala Val Asp Gly Val Arg Phe Ile Ala Asp His Met Arg Ser Glu 430 435 440	1587
GAC GAT GAC CAG AGC GTG AGT GAG GAC TGG AAG TAC GTC GCC ATG GTG Asp Asp Asp Gln Ser Val Ser Glu Asp Trp Lys Tyr Val Ala Met Val 445 450 455	1635
ATC GAC CGC CTC TTC CTC TGG ATC TTT GTC TTT GTC TGT GTC TTT GGC Ile Asp Arg Leu Phe Leu Trp Ile Phe Val Phe Val Cys Val Phe Gly 460 465 470	1683
ACC ATC GGC ATG TTC CTG CAG CCT CTC TTC CAG AAC TAC ACC ACC ACC Thr Ile Gly Met Phe Leu Gln Pro Leu Phe Gln Asn Tyr Thr Thr Thr 475 480 485	1731
ACC TTC CTC CAC TCA GAC CAC TCA GCC CCC AGC TCC AAG TGAGGCCCTT Thr Phe Leu His Ser Asp His Ser Ala Pro Ser Ser Lys 490 495 500	1780
CCTCATCTCC ATGCTCTTTC ACCCTGCCAC CCTCTGCTGC ACAGTAGTGT TGGGTGGAGG	1840
ATGGACGAGT GAGCTACCAG GAAGAGGGGC GCTGCCCCCA CAGATCCATC CTTTGTCTTC	1900
ATCTGGAGTC CCTCCTCCCC CACGCCTCCA TCCACACACA GCAGCTCCAA CCTGGAGGCT	1960
GGACCAACTG CTTTGTCTTC GCTGCTCTCC ATCTCTTGTA CCAGCCCAGG CAATAGTGTT	2020
GAGGAGGGGA GCAAGGCTGC TAAGTGAAG ACAGAGATGG CAGAGCCATC CACCCTGAGG	2080
AGTGACGGGC AAGGGGCCAG GAAGGGGACA GGATTGTCTG CTGCCTCCAA GTCATGGGAG	2140
AAGAGGGGTA TAGGACAAGG GGTGGAAGGG CAGGAGCTCA CACCGCACCG GGCTGGCCTG	2200
ACACAATGGT AGCTCTGAAG GGAGGGGAAG AGAGAGGCCT GGGTGTGACC TGACACCTGC	2260
CGTGCTTGA GTGGACAGCA GCTGGACTGG GTGGGCCCCA CAGTGGTCAG CGATTCTGTC	2320
CAAGTAGGGT TTAGCCGGGC CCCATGGTCA CAGACCCCTG GGGGAGGCTT CCAGCTCACT	2380
CCCACAGCCC CTTGCTTCTA AGGGATCCAG AGACCTGCTC CAGATCCTCT TTCCCCACTG	2440
AAGAATTC	2448

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 502 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met	Ala	Arg	Arg	Cys	Gly	Pro	Val	Ala	Leu	Leu	Gly	Phe	Gly	Leu
1				5					10				15	
Leu	Arg	Leu	Cys	Ser	Gly	Val	Trp	Gly	Thr	Asp	Thr	Glu	Glu	Arg
		20						25				30		Leu
Val	Glu	His	Leu	Leu	Asp	Pro	Ser	Arg	Tyr	Asn	Lys	Leu	Ile	Arg
		35					40				45			Pro
Ala	Thr	Asn	Gly	Ser	Glu	Leu	Val	Thr	Val	Gln	Leu	Met	Val	Ser
	50						55				60			Leu

Ala	Gln	Leu	Ile	Ser	Val	His	Glu	Arg	Glu	Gln	Ile	Met	Thr	Thr	Asn	
65					70					75					80	
Val	Trp	Leu	Thr	Gln	Glu	Trp	Glu	Asp	Tyr	Arg	Leu	Thr	Trp	Lys	Pro	
				85					90					95		
Glu	Glu	Phe	Asp	Asn	Met	Lys	Lys	Val	Arg	Leu	Pro	Ser	Lys	His	Ile	
			100					105					110			
Trp	Leu	Pro	Asp	Val	Val	Leu	Tyr	Asn	Asn	Ala	Asp	Gly	Met	Tyr	Glu	
		115					120					125				
Val	Ser	Phe	Tyr	Ser	Asn	Ala	Val	Val	Ser	Tyr	Asp	Gly	Ser	Ile	Phe	
	130					135					140					
Trp	Leu	Pro	Pro	Ala	Ile	Tyr	Lys	Ser	Ala	Cys	Lys	Ile	Glu	Val	Lys	
145					150					155					160	
His	Phe	Pro	Phe	Asp	Gln	Gln	Asn	Cys	Thr	Met	Lys	Phe	Arg	Ser	Trp	
				165					170					175		
Thr	Tyr	Asp	Arg	Thr	Glu	Ile	Asp	Leu	Val	Leu	Lys	Ser	Glu	Val	Ala	
			180					185					190			
Ser	Leu	Asp	Asp	Phe	Thr	Pro	Ser	Gly	Glu	Trp	Asp	Ile	Val	Ala	Leu	
		195					200					205				
Pro	Gly	Arg	Arg	Asn	Glu	Asn	Pro	Asp	Asp	Ser	Thr	Tyr	Val	Asp	Ile	
	210					215					220					
Thr	Tyr	Asp	Phe	Ile	Ile	Arg	Arg	Lys	Pro	Leu	Phe	Tyr	Thr	Ile	Asn	
225					230					235					240	
Leu	Ile	Ile	Pro	Cys	Val	Leu	Ile	Thr	Ser	Leu	Ala	Ile	Leu	Val	Phe	
				245					250				255			
Tyr	Leu	Pro	Ser	Asp	Cys	Gly	Glu	Lys	Met	Thr	Leu	Cys	Ile	Ser	Val	
			260					265					270			
Leu	Leu	Ala	Leu	Thr	Val	Phe	Leu	Leu	Leu	Ile	Ser	Lys	Ile	Val	Pro	
		275					280					285				
Pro	Thr	Ser	Leu	Asp	Val	Pro	Leu	Val	Gly	Lys	Tyr	Leu	Met	Phe	Thr	
	290					295					300					
Met	Val	Leu	Val	Thr	Phe	Ser	Ile	Val	Thr	Ser	Val	Cys	Val	Leu	Asn	
305					310					315					320	
Val	His	His	Arg	Ser	Pro	Thr	Thr	His	Thr	Met	Ala	Pro	Trp	Val	Lys	
				325					330					335		
Val	Val	Phe	Leu	Glu	Lys	Leu	Pro	Ala	Leu	Leu	Phe	Met	Gln	Gln	Pro	
			340					345					350			
Arg	His	His	Cys	Ala	Arg	Gln	Arg	Leu	Arg	Leu	Arg	Arg	Arg	Gln	Arg	
		355					360					365				
Glu	Arg	Glu	Gly	Ala	Gly	Ala	Leu	Phe	Phe	Arg	Glu	Ala	Pro	Gly	Ala	
	370					375					380					
Asp	Ser	Cys	Thr	Cys	Phe	Val	Asn	Arg	Ala	Ser	Val	Gln	Gly	Leu	Ala	
385					390					395					400	
Gly	Ala	Phe	Gly	Ala	Glu	Pro	Ala	Pro	Val	Ala	Gly	Pro	Gly	Arg	Ser	
				405					410					415		

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Gly Glu Pro Cys Gly Cys Gly Leu Arg Glu Ala Val Asp Gly Val Arg  
420 425 430  
Phe Ile Ala Asp His Met Arg Ser Glu Asp Asp Asp Gln Ser Val Ser  
435 440 445  
Glu Asp Trp Lys Tyr Val Ala Met Val Ile Asp Arg Leu Phe Leu Trp  
450 455 460  
Ile Phe Val Phe Val Cys Val Phe Gly Thr Ile Gly Met Phe Leu Gln  
465 470 475 480  
Pro Leu Phe Gln Asn Tyr Thr Thr Thr Thr Phe Leu His Ser Asp His  
485 490 495  
Ser Ala Pro Ser Ser Lys  
500

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1927 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 98..1474
- (D) OTHER INFORMATION: /product= "BETA-3 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCGGAACCCC TGTATTTTCT TTTCAAAACC CCCTTTTCCA GTGGAAATGC TCTGTTGTTA	60
AAAAGGAAGA AACTGTCTTT CTGAAACTGA CATCACG ATG CTC CCA GAT TTT ATG	115
Met Leu Pro Asp Phe Met	5
CTG GTT CTC ATC GTC CTT GGC ATC CCT TCC TCA GCC ACC ACA GGT TTC	163
Leu Val Leu Ile Val Leu Gly Ile Pro Ser Ser Ala Thr Thr Gly Phe	20
AAC TCA ATC GCC GAA AAT GAA GAT GCC CTC CTC AGA CAT TTG TTC CAA	211
Asn Ser Ile Ala Glu Asn Glu Asp Ala Leu Leu Arg His Leu Phe Gln	35
GGT TAT CAG AAA TGG GTC CGC CCT GTA TTA CAT TCT AAT GAC ACC ATA	259
Gly Tyr Gln Lys Trp Val Arg Pro Val Leu His Ser Asn Asp Thr Ile	50
AAA GTA TAT TTT GGA TTG AAA ATA TCC CAG CTT GTA GAT GTG GAT GAA	307
Lys Val Tyr Phe Gly Leu Lys Ile Ser Gln Leu Val Asp Val Asp Glu	70
AAG AAT CAG CTG ATG ACA ACC AAT GTG TGG CTC AAA CAG GAA TGG ACA	355
Lys Asn Gln Leu Met Thr Thr Asn Val Trp Leu Lys Gln Glu Trp Thr	85
GAC CAC AAG TTA CGC TGG AAT CCT GAT GAT TAT GGT GGG ATC CAT TCC	403
Asp His Lys Leu Arg Trp Asn Pro Asp Asp Tyr Gly Gly Ile His Ser	100

ATT AAA GTT CCA TCA GAA TCT CTG TGG CTT CCT GAC ATA GTT CTC TTT Ile Lys Val Pro Ser Glu Ser Leu Trp Leu Pro Asp Ile Val Leu Phe 105 110 115	451
GAA AAT GCT GAC GGC CGC TTC GAA GGC TCC CTG ATG ACC AAG GTC ATC Glu Asn Ala Asp Gly Arg Phe Glu Gly Ser Leu Met Thr Lys Val Ile 120 125 130	499
GTG AAA TCA AAC GGA ACT GTT GTC TGG ACC CCT CCC GCC AGC TAC AAA Val Lys Ser Asn Gly Thr Val Val Trp Thr Pro Pro Ala Ser Tyr Lys 135 140 145 150	547
AGC TCC TGC ACC ATG GAC GTC ACG TTT TTC CCG TTC GAC CGA CAG AAC Ser Ser Cys Thr Met Asp Val Thr Phe Phe Pro Phe Asp Arg Gln Asn 155 160 165	595
TGC TCC ATG AAG TTT GGA TCC TGG ACT TAT GAT GGC ACC ATG GTT GAC Cys Ser Met Lys Phe Gly Ser Trp Thr Tyr Asp Gly Thr Met Val Asp 170 175 180	643
CTC ATT TTG ATC AAT GAA AAT GTC GAC AGA AAA GAC TTC TTC GAT AAC Leu Ile Leu Ile Asn Glu Asn Val Asp Arg Lys Asp Phe Phe Asp Asn 185 190 195	691
GGA GAA TGG GAA ATA CTG AAT GCA AAG GGG ATG AAG GGG AAC AGA AGG Gly Glu Trp Glu Ile Leu Asn Ala Lys Gly Met Lys Gly Asn Arg Arg 200 205 210	739
GAC GGC GTG TAC TCC TAT CCC TTT ATC ACG TAT TCC TTC GTC CTG AGA Asp Gly Val Tyr Ser Tyr Pro Phe Ile Thr Tyr Ser Phe Val Leu Arg 215 220 225 230	787
CGC CTG CCT TTA TTC TAT ACC CTC TTT CTC ATC ATC CCC TGC CTG GGG Arg Leu Pro Leu Phe Tyr Thr Leu Phe Leu Ile Ile Pro Cys Leu Gly 235 240 245	835
CTG TCT TTC CTA ACA GTT CTT GTG TTC TAT TTA CCT TCG GAT GAA GGA Leu Ser Phe Leu Thr Val Leu Val Phe Tyr Leu Pro Ser Asp Glu Gly 250 255 260	883
GAA AAA CTT TCA TTA TCC ACA TCG GTC TTG GTT TCT CTG ACA GTT TTC Glu Lys Leu Ser Leu Ser Thr Ser Val Leu Val Ser Leu Thr Val Phe 265 270 275	931
CTT TTA GTG ATT GAA GAA ATC ATC CCA TCG TCT TCC AAA GTC ATT CCT Leu Leu Val Ile Glu Glu Ile Ile Pro Ser Ser Ser Lys Val Ile Pro 280 285 290	979
CTC ATT GGA GAG TAC CTG CTG TTC ATC ATG ATT TTT GTG ACC CTG TCC Leu Ile Gly Glu Tyr Leu Leu Phe Ile Met Ile Phe Val Thr Leu Ser 295 300 305 310	1027
ATC ATT GTT ACC GTG TTT GTC ATT AAC GTT CAC CAC AGA TCT TCT TCC Ile Ile Val Thr Val Phe Val Ile Asn Val His His Arg Ser Ser Ser 315 320 325	1075
ACG TAC CAC CCC ATG GCC CCC TGG GTT AAG AGG CTC TTT CTG CAG AAA Thr Tyr His Pro Met Ala Pro Trp Val Lys Arg Leu Phe Leu Gln Lys 330 335 340	1123
CTT CCA AAA TTA CTT TGC ATG AAA GAT CAT GTG GAT CGC TAC TCA TCC Leu Pro Lys Leu Leu Cys Met Lys Asp His Val Asp Arg Tyr Ser Ser 345 350 355	1171
CCA GAG AAA GAG GAG AGT CAA CCA GTA GTG AAA GGC AAA GTC CTC GAA Pro Glu Lys Glu Glu Ser Gln Pro Val Val Lys Gly Lys Val Leu Glu 360 365 370	1219

AAA AAG AAA CAG AAA CAG CTT AGT GAT GGA GAA AAA GTT CTA GTT GCT Lys Lys Lys Gln Lys Gln Leu Ser Asp Gly Glu Lys Val Leu Val Ala 375 380 385 390	1267
TTT TTG GAA AAA GCT GCT GAT TCC ATT AGA TAC ATT TCC AGA CAT GTG Phe Leu Glu Lys Ala Ala Asp Ser Ile Arg Tyr Ile Ser Arg His Val 395 400 405	1315
AAG AAA GAA CAT TTT ATC AGC CAG GTA GTA CAA GAC TGG AAA TTT GTA Lys Lys Glu His Phe Ile Ser Gln Val Val Gln Asp Trp Lys Phe Val 410 415 420	1363
GCT CAA GTT CTT GAC CGA ATC TTC CTG TGG CTC TTT CTG ATA GTG TCA Ala Gln Val Leu Asp Arg Ile Phe Leu Trp Leu Phe Leu Ile Val Ser 425 430 435	1411
GCA ACA GGC TCG GTT CTG ATT TTT ACC CCT GCT TTG AAG ATG TGG CTA Ala Thr Gly Ser Val Leu Ile Phe Thr Pro Ala Leu Lys Met Trp Leu 440 445 450	1459
CAT AGT TAC CAT TAGGAATTTT AAAAGACATA AGTACTAAAT TACACCTTAG His Ser Tyr His 455	1511
ACCTGACATC TGGCTATCAC ACAGACAGAA TCCAAATGCA TGTGCTTGTT CTACGAACCC	1571
CGAATGCGTT GTCTTTGTGG AAATGGAACA TCTCCTCATG GGAGAACTC TGGTAAATGT	1631
GCTCATTGT GGTGCCATG AGAGTGAGCT GCTTTTAAAG AAAGTGGAGC CTCCTCAGAC	1691
CCCTGCCTTG GCTTTCCAG ACATTGAGGG AGGGATCATA GGTCCAGGCT TGAGCTCACA	1751
TGTGGCCAGA GTGCACAAA AGCTGTGCT ACTTGGTCCA GGAACACCTC CTAGAAGCAG	1811
CAGGCCTCGG TGGTGGGGGA GGGGGGATTC ACCTGGAATT AAGGAAGTCT CGGTGTCCAG	1871
CTATCTGTGT GGGCAGAGCC TGGATCTCCC ACCCTGCACT GGCTCCTTG GTGCCG	1927

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 458 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Leu Pro Asp Phe Met Leu Val Leu Ile Val Leu Gly Ile Pro Ser 1 5 10 15
Ser Ala Thr Thr Gly Phe Asn Ser Ile Ala Glu Asn Glu Asp Ala Leu 20 25 30
Leu Arg His Leu Phe Gln Gly Tyr Gln Lys Trp Val Arg Pro Val Leu 35 40 45
His Ser Asn Asp Thr Ile Lys Val Tyr Phe Gly Leu Lys Ile Ser Gln 50 55 60
Leu Val Asp Val Asp Glu Lys Asn Gln Leu Met Thr Thr Asn Val Trp 65 70 75 80
Leu Lys Gln Glu Trp Thr Asp His Lys Leu Arg Trp Asn Pro Asp Asp 85 90 95



Tyr Gly Gly Ile His Ser Ile Lys Val Pro Ser Glu Ser Leu Trp Leu  
 100 105 110  
 Pro Asp Ile Val Leu Phe Glu Asn Ala Asp Gly Arg Phe Glu Gly Ser  
 115 120 125  
 Leu Met Thr Lys Val Ile Val Lys Ser Asn Gly Thr Val Val Trp Thr  
 130 135 140  
 Pro Pro Ala Ser Tyr Lys Ser Ser Cys Thr Met Asp Val Thr Phe Phe  
 145 150 155 160  
 Pro Phe Asp Arg Gln Asn Cys Ser Met Lys Phe Gly Ser Trp Thr Tyr  
 165 170 175  
 Asp Gly Thr Met Val Asp Leu Ile Leu Ile Asn Glu Asn Val Asp Arg  
 180 185 190  
 Lys Asp Phe Phe Asp Asn Gly Glu Trp Glu Ile Leu Asn Ala Lys Gly  
 195 200 205  
 Met Lys Gly Asn Arg Arg Asp Gly Val Tyr Ser Tyr Pro Phe Ile Thr  
 210 215 220  
 Tyr Ser Phe Val Leu Arg Arg Leu Pro Leu Phe Tyr Thr Leu Phe Leu  
 225 230 235 240  
 Ile Ile Pro Cys Leu Gly Leu Ser Phe Leu Thr Val Leu Val Phe Tyr  
 245 250 255  
 Leu Pro Ser Asp Glu Gly Glu Lys Leu Ser Leu Ser Thr Ser Val Leu  
 260 265 270  
 Val Ser Leu Thr Val Phe Leu Leu Val Ile Glu Glu Ile Ile Pro Ser  
 275 280 285  
 Ser Ser Lys Val Ile Pro Leu Ile Gly Glu Tyr Leu Leu Phe Ile Met  
 290 295 300  
 Ile Phe Val Thr Leu Ser Ile Ile Val Thr Val Phe Val Ile Asn Val  
 305 310 315 320  
 His His Arg Ser Ser Ser Thr Tyr His Pro Met Ala Pro Trp Val Lys  
 325 330 335  
 Arg Leu Phe Leu Gln Lys Leu Pro Lys Leu Leu Cys Met Lys Asp His  
 340 345 350  
 Val Asp Arg Tyr Ser Ser Pro Glu Lys Glu Glu Ser Gln Pro Val Val  
 355 360 365  
 Lys Gly Lys Val Leu Glu Lys Lys Lys Gln Lys Gln Leu Ser Asp Gly  
 370 375 380  
 Glu Lys Val Leu Val Ala Phe Leu Glu Lys Ala Ala Asp Ser Ile Arg  
 385 390 395 400  
 Tyr Ile Ser Arg His Val Lys Lys Glu His Phe Ile Ser Gln Val Val  
 405 410 415  
 Gln Asp Trp Lys Phe Val Ala Gln Val Leu Asp Arg Ile Phe Leu Trp  
 420 425 430  
 Leu Phe Leu Ile Val Ser Ala Thr Gly Ser Val Leu Ile Phe Thr Pro  
 435 440 445

Ala Leu Lys Met Trp Leu His Ser Tyr His  
450 455

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1915 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 87..1583
- (D) OTHER INFORMATION: /product= "BETA-4 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCGGCGCTCA CTCGACCGCG CGGCTCACGG GTGCCCTGTG ACCCCACAGC GGAGCTCGCG	60
GCGGCTGCCA CCGGGCCCCG CCGGCCATGA GCGCGCGGCC TTCCCTGGTC CTTTCTTCC	120
TGGTCGCCCT TTGCGGGCGC GGGAACTGCC GCGTGGCCAA TGCGGAGGAA AAGCTGATGG	180
ACGACCTTCT GAACAAAACC CGTTACAATA ACCTGATCCG CCCAGCCACC AGCTCCTCAC	240
AGCTCATCTC CATCAAGCTG CAGCTCTCCC TGGCCCAGCT TATCAGCGTG AATGAGCGAG	300
AGCAGATCAT GACCACCAAT GTCTGGCTGA AACAGGAATG GACTGATTAC CGCCTGACCT	360
GGAACAGCTC CCGCTACGAG GGTGTGAACA TCCTGAGGAT CCCTGCAAAG CGCATCTGGT	420
TGCCTGACAT CGTGCTTTAC AACAAACGCC ACGGGACCTA TGAGGTGTCT GTCTACACCA	480
ACTTGATAGT CCGGTCCAAC GGCAGCGTCC TGTGGCTGCC CCCTGCCATC TACAAGAGCG	540
CCTGCAAGAT TGAGGTGAAG TACTTTCCCT TCGACCAGCA GAACTGCACC CTCAAGTTCC	600
GCTCCTGGAC CTATGACCAC ACGGAGATAG ACATGGTCCT CATGACGCCC ACAGCCAGCA	660
TGGATGACTT TACTCCAGT GGTGAGTGGG ACATAGTGGC CCTCCCAGGG AGAAGGACAG	720
TGAACCCACA AGACCCAGC TACGTGGAGC TGACTTACGA CTTTCATATC AAGCGCAAGC	780
CTCTGTTCTA CACCATCAAC CTCATCATCC CCTGCGTGCT CACCACCTTG CTGGCCATCC	840
TCGTCTTCTA CCTGCCATCC GACTGCGGCG AGAAGATGAC ACTGTGCATC TCAGTGCTGC	900
TGGCACTGAC ATTCTTCCTG CTGCTCATCT CCAAGATCGT GCCACCCACC TCCCTCGATG	960
TGCCTCTCAT CGGCAAGTAC CTCATGTTCA CCATGGTGCT GGTCACTTC TCCATCGTCA	1020
CCAGCGTCTG TGTGCTCAAT GTGCACCACC GCTCGCCCAG CACCCACACC ATGGCACCCCT	1080
GGGTCAAGCG CTGCTTCCTG CACAAGCTGC CTACCTTCCT CTTTCATGAAG CGCCCTGGCC	1140
CCGACAGCAG CCGGGCCAGA GCCTTCCCGC CCAGCAAGTC ATGCGTGACC AAGCCCGAGG	1200
CCACCGCCAC CTCCACCAGC CCCTCCAAC TCTATGGGAA CTCCATGTAC TTTGTGAACC	1260
CCGCTCTGCG AGCTTCCAAG TCTCCAGCCG GCTCTACCCC GGTGGCTATC CCCAGGGATT	1320
TCTGGCTGCG GTCTCTGGG AGGTTCCGAC AGGATGTGCA GGAGGCATTA GAAGGTGTCA	1380

GCTTCATCGC CCAGCACATG AAGAATGACG ATGAAGACCA GAGTGTGCTT GAGGACTGGA 1440  
 AGTACGTGGC TATGGTGGTG GACCGGCTGT TCCTGTGGGT GTTCATGTTT GTGTGCGTCC 1500  
 TGGGCACTGT GGGGCTCTTC CTGCCGCCCC TCTTCAGAC CCATGCAGCT TCTGAGGGGC 1560  
 CCTACGCTGC CCAGCGTGAC TGAGGGCCCC CTGGGTGTG GGGTGAGAGG ATGTGAGTGG 1620  
 CCGGGTGGGC ACTTTGCTGC TTCTTTCTGG GTTGTGGCCG ATGAGGCCCT AAGTAAATAT 1680  
 GTGAGCATTG GCCATCAACC CCATCAAACC AGCCACAGCC GTGGAACAGG CAAGGATGGG 1740  
 GGCCTGGCCT GTCTCTCTG AATGCCTTGG AGGGATCCCA GGAAGCCCCA GTAGGAGGGA 1800  
 GCTTCAGACA GTTCAATTCT GGCCTGTCTT CCTTCCCTGC ACCGGGCAAT GGGGATAAAG 1860  
 ATGACTTCGT AGCAGCACCT ACTATGCTTC AGGCATGGTG CCGGCCTGCC TCTCC 1915

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 498 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met	Arg	Arg	Ala	Pro	Ser	Leu	Val	Leu	Phe	Phe	Leu	Val	Ala	Leu	Cys
1				5					10					15	
Gly	Arg	Gly	Asn	Cys	Arg	Val	Ala	Asn	Ala	Glu	Glu	Lys	Leu	Met	Asp
			20					25					30		
Asp	Leu	Leu	Asn	Lys	Thr	Arg	Tyr	Asn	Asn	Leu	Ile	Arg	Pro	Ala	Thr
			35				40					45			
Ser	Ser	Ser	Gln	Leu	Ile	Ser	Ile	Lys	Leu	Gln	Leu	Ser	Leu	Ala	Gln
			50			55				60					
Leu	Ile	Ser	Val	Asn	Glu	Arg	Glu	Gln	Ile	Met	Thr	Thr	Asn	Val	Trp
65				70					75					80	
Leu	Lys	Gln	Glu	Trp	Thr	Asp	Tyr	Arg	Leu	Thr	Trp	Asn	Ser	Ser	Arg
			85						90					95	
Tyr	Glu	Gly	Val	Asn	Ile	Leu	Arg	Ile	Pro	Ala	Lys	Arg	Ile	Trp	Leu
			100				105						110		
Pro	Asp	Ile	Val	Leu	Tyr	Asn	Asn	Ala	Asp	Gly	Thr	Tyr	Glu	Val	Ser
			115				120					125			
Val	Tyr	Thr	Asn	Leu	Ile	Val	Arg	Ser	Asn	Gly	Ser	Val	Leu	Trp	Leu
			130			135					140				
Pro	Pro	Ala	Ile	Tyr	Lys	Ser	Ala	Cys	Lys	Ile	Glu	Val	Lys	Tyr	Phe
145				150					155						160
Pro	Phe	Asp	Gln	Gln	Asn	Cys	Thr	Leu	Lys	Phe	Arg	Ser	Trp	Thr	Tyr
			165						170					175	
Asp	His	Thr	Glu	Ile	Asp	Met	Val	Leu	Met	Thr	Pro	Thr	Ala	Ser	Met
			180				185						190		
Asp	Asp	Phe	Thr	Pro	Ser	Gly	Glu	Trp	Asp	Ile	Val	Ala	Leu	Pro	Gly
			195				200					205			

Arg Arg Thr Val Asn Pro Gln Asp Pro Ser Tyr Val Asp Val Thr Tyr  
 210 215 220  
 Asp Phe Ile Ile Lys Arg Lys Pro Leu Phe Tyr Thr Ile Asn Leu Ile  
 225 230 235 240  
 Ile Pro Cys Val Leu Thr Thr Leu Leu Ala Ile Leu Val Phe Tyr Leu  
 245 250 255  
 Pro Ser Asp Cys Gly Glu Lys Met Thr Leu Cys Ile Ser Val Leu Leu  
 260 265 270  
 Ala Leu Thr Phe Phe Leu Leu Leu Ile Ser Lys Ile Val Pro Pro Thr  
 275 280 285  
 Ser Leu Asp Val Pro Leu Ile Gly Lys Tyr Leu Met Phe Thr Met Val  
 290 295 300  
 Leu Val Thr Phe Ser Ile Val Thr Ser Val Cys Val Leu Asn Val His  
 305 310 315 320  
 His Arg Ser Pro Ser Thr His Thr Met Ala Pro Trp Val Lys Arg Cys  
 325 330 335  
 Phe Leu His Lys Leu Pro Thr Phe Leu Phe Met Lys Arg Pro Gly Pro  
 340 345 350  
 Asp Ser Ser Pro Ala Arg Ala Phe Pro Pro Ser Lys Ser Cys Val Thr  
 355 360 365  
 Lys Pro Glu Ala Thr Ala Thr Ser Thr Ser Pro Ser Asn Phe Tyr Gly  
 370 375 380  
 Asn Ser Met Tyr Phe Val Asn Pro Ala Ser Ala Ala Ser Lys Ser Pro  
 385 390 395 400  
 Ala Gly Ser Thr Pro Val Ala Ile Pro Arg Asp Phe Trp Leu Arg Ser  
 405 410 415  
 Ser Gly Arg Phe Arg Gln Asp Val Gln Glu Ala Leu Glu Gly Val Ser  
 420 425 430  
 Phe Ile Ala Gln His Met Lys Asn Asp Asp Glu Asp Gln Ser Val Val  
 435 440 445  
 Glu Asp Trp Lys Tyr Val Ala Met Val Val Asp Arg Leu Phe Leu Trp  
 450 455 460  
 Val Phe Met Phe Val Cys Val Leu Gly Thr Val Gly Leu Phe Leu Pro  
 465 470 475 480  
 Pro Leu Phe Gln Thr His Ala Ala Ser Glu Gly Pro Tyr Ala Ala Gln  
 485 490 495  
 Arg Asp